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Characterisation of the inhibitory EP-receptors present in human monocytes

by
Stuart Angus Milne

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ABSTRACT

PGE_2 has been shown to possess both pro- and anti-inflammatory properties. Four subtypes of EP-receptor, termed EP_1, EP_2, EP_3 and EP_4 have been identified pharmacologically and have been cloned in the human.

The aim of this thesis was twofold. Initial studies involved confirmation and further characterisation of the newly described EP_4 receptor subtype. For this purpose, a range of agonists and, where possible, antagonists were investigated using the Chinese hamster ovary (CHO) cells, pig saphenous vein (PSV) and rabbit jugular vein (RJV) preparations. The second and primary aim of this thesis was to classify the EP receptor subtype present in human monocytes.

PGE_2 has been shown to have inhibitory effects on human monocyte/macrophages which are likely to be mediated via the EP_2 and/or EP_4 receptors as these receptors have been associated with relaxation and down-regulation. Little information has been published with respect to the EP_4 receptor, our aim was to use data on the EP_4 receptor obtained in the first part of this thesis along with already published data on the EP_2 receptor, to classify the inhibitory EP receptor(s) expressed on human monocytes.

Our results showed that the native CHO cell line expresses EP_4 receptors positively coupled to AC and demonstrated further that the PSV is an EP_4 receptor containing preparation. Studies with the RJV, however, do not identify either an EP_2 or an EP_4 receptor containing preparation. Studies with the human monocyte indicated that both EP_2 and EP_4 receptors are expressed. The studies measuring production of cAMP showed that predominately EP_4 receptors mediate the activation of AC. Work examining the functional response of PGE_2 at inhibiting formyl-Met-Leu-Phe (FMLP)-induced superoxide anion generation demonstrated a predominately EP_2 receptor mediated response. Second messenger studies investigating the inhibition of FMLP-induced superoxide anion generation by PGE_2 indicated that this was via a non-cAMP-dependent pathway.

Butaprost has been important in the classification of the EP_2 and EP_4 receptor subtypes. Interestingly, butaprost showed no affinity for the cloned EP_4 receptor, whereas it demonstrated full agonist activity at the cloned EP_2 receptor. The observation that butaprost induces complete relaxation to the PSV, an EP_4 receptor containing preparation, suggests that there is co-expression of EP_2 and EP_4 receptors in this preparation. This co-expression has also been demonstrated here in the human monocyte and may be the case in other experimental preparations.

The EP_2 receptor has also been shown to mediate the effects of PGE_2 in human neutrophils and eosinophils. Since all these leukocytes express the EP_2 receptor this may limit the potential for the development of EP-agonists as selective inhibitors of monocyte function.

I declare that this thesis has been composed by myself and the research has been conducted solely by me.

Stuart Milne

ACKNOWLEDGEMENT

I wish to thank my supervisor, Dr. Roma Armstrong, for her assistance, advice and patience throughout my PhD. I particularly wish to thank Roma for taking me on as a PhD student at a very difficult stage in my career.

I wish to thank my second supervisor, Dr. David Woodward, for agreeing to fund the project and also for his assistance throughout the work.

I would especially like to thank my parents as, without their help, I would not have been able to do this PhD in the first place.

Finally, I wish to thank my wife for her patience and help throughout the writing of my thesis. It has been Elodie's support that has kept me going.

PUBLICATIONS

Full papers

MILNE, S.A., ARMSTRONG, R.A. & WOODWARD, D.F. (1995). Investigation of prostanoid EP receptors in rabbit jugular and pig saphenous veins. *Prostaglandins*, **49**, 225-237.

Abstracts

MILNE, S.A., SALA TENNA, A.M., ARMSTRONG, R.A. & WOODWARD, D.F. (1994). Further investigation of prostanoid EP receptors in rabbit jugular and pig saphenous veins. *Br. J. Pharmacol.*, **111**, 79P.

MILNE, S.A., LEE, J., ARMSTRONG, R.A. & WOODWARD, D.F. (1994). Human monocytes and CHO cells both contain EP_4 receptors coupled to adenylate cyclase. *Br. J. Pharmacol*, **113**, 8P.

MILNE, S.A., & ARMSTRONG, R. (1995). Effect of the prostaglandin E agonist, nocloprost, on human monocyte cyclic AMP production and superoxide anion generation. *EPHAR, 1995.*

ABBREVIATIONS

5-HT	5-hydroxy tryptamine
AA	arachidonic acid
AC	adenylate cyclase
AChE	acetylcholinesterase
ANOVA	analysis of variance
BSA	bovine serum albumin
C5a	complement 5a
cAMP	adenosine 3':5'-cyclic monophosphate
cGMP	guanine 3':5'-cyclic monophosphate
CHO	chinese hamster ovary
COX	cyclooxygenase
CSF	colony stimulating factor
CT	cholera toxin
DAG	diacylglycerol
DARS	donkey anti-rabbit serum
DEPC	diethyl pyrocarbonate
DMSO	dimethyl sulphoxide
DNA	deoxyribonucleic acid
EAE	experimental allergic encephalomyelitis
EEC	equi-effective concentration
ELISA	enzyme linked immunosorbant assay
FCS	foetal calf serum
FMLP	formyl-methionyl-leucyl-phenylalanine
G-CSF	granulocyte colony stimulating factor
GM-CSF	granulocyte macrophage colony stimulating factor
GIT	guanidium isothiocyanate
H_2O_2	hydrogen peroxide
HBSS	Hanks' balanced salt solution
HIV	human immunodeficiency virus
IBMX	isobutyl methylxanthine
ICE	interleukin converting enzyme
IL-1α	interleukin 1α
IL-1β	interleukin 1β
IL-1ra	interleukin receptor antagonist
INFγ	interferon γ

IOP	intraocular pressure
IP_3	inositol 1,4,5-trisphosphate
LDL	low-density lipoprotein
L-NMMA	L-N^G-monomethyl arginine
LPS	lipopolysaccharide
LTB_4	leukotriene B_4
M-CSF	monocyte colony stimulating factor
MMP	matrix metalloproteinases
mRNA	messenger RNA
MS	multiple sclerosis
NRS	normal rabbit serum
NSB	non-specific binding
O_2^-	superoxide anion
PAF	platelet activating factor
PBS	phosphate buffer saline
PDBu	12,13-phorbol dibutyrate
PDE	phosphodiesterase
PEG	polyethylene glycol
PG	prostaglandin
PGA_2	prostaglandin A_2
PGB_2	prostaglandin B_2
PGC_2	prostaglandin C_2
PGD_2	prostaglandin D_2
PGE_1	prostaglandin E_1
PGE_2	prostaglandin E_2
$PGF_{1\alpha}$	prostaglandin $F_{1\alpha}$
$PGF_{2\alpha}$	prostaglandin $F_{2\alpha}$
PGI_2	prostaglandin I_2
PKA	protein kinase A
PKC	protein kinase C
PLA_2	phospholiapse A_2
PLC	phospholipase C
PMA	phorbol myristal acetate
PMNC	polymorphonuclear cells
PSV	pig saphenous vein
PT	pertussis toxin
RIA	radioimmunoassay

RJV	rabbit jugular vein
RNA	ribonucleic acid
SEM	standard error of the mean
SOD	superoxide dismutase
TNFα	tumour necrosis factor-α
TXA_2	thromboxane A_2

CHEMICAL NAMES

AH13205- trans-2[4-(1-hydroxyhexyl)pentyl)phenyl]-5-oxocyclo pentaneheptanoic acid

AH23848B- 9[1α(Z), 2β, 5α]-(±)-7-[5-[[(1,1'-biphenyl)-4-yl]methoxyl]-2-(4-morpho-linyl)-3-oxocyclopentyl]-4-heptanoic acid)

AH6809- 6-iso-propoxy-9-oxaxanthene-2-carboxylic acid

GR32191X- [1R-[1α(Z)2β,3α,5α]]-(+)-7-[5-[[(1,1'-biphenyl)-4-yl]methoxy]-3-hydroxy-2-(1-piperidinyl)cyclopentyl]-4-heptenoic acid

GR63799X- [1R-[1α(Z)2β(R^s),3α]-(-)-4-benzoylamino)phenyl-7-[3-hydroxy-3-phenoxypropoxy)-5-oxocyclopentyl]-4-heptenoate

M&B28,767- 15S-hydroxy-9-oxo-16-phenoxy-ω-tetranorprost-13E-enoic acid

SQ22,536- ((9-tetrahydro-2-furyl)adenine

COMPOUNDS

The following compounds were gifts which we gratefully acknowledge:

1° antibody for the RIA	Dr B. Williams, Edinburgh University
^{125}I-cAMP	Dr F. Antoni, Edinburgh University
AH13205	Dr B. Bain, Glaxo, UK
AH23848B	Dr B. Bain, Glaxo, UK
AH6809	Dr B. Bain, Glaxo, UK
butaprost	Dr P. Gardiner, Bayer, UK
BW A868C	Dr B. Whittle, Wellcome, UK
GR32191X	Dr B. Bain, Glaxo, UK
GR63799X	Dr B. Bain, Glaxo, UK
L-NMMA	Dr B. Whittle, Wellcome, UK
M&B28767	Dr M. Caton, Rhone-Poulenc, UK
misoprostol	Dr P. Collins, G.D. Searle, USA
nocloprost	Dr E. Schillinger, Schering AG, Berlin
rolipram	Dr E. Schillinger, Schering AG, Berlin
SQ22,536	(made in department) Dr N. H. Wilson.
sulprostone	Dr E. Schillinger, Schering AG, Berlin

The materials used below were obtained from the following sources

11-deoxy PGE_1	Cayman Chemicals
16,16-dimethyl PGE_2	Cayman Chemicals
17-phenyl-γ-trinor PGE_2	Cayman Chemicals
BSA	Aldrich
DMSO	Sigma
ELISA kits	Cayman Chemicals
ferricytochrome C	Sigma
FCS	Gibco
FMLP	Sigma
HAMS F-12	Gibco
HBSS	Gibco
Heparin	CP Pharmaceuticals Ltd.
IBMX	Sigma
indomethacin	Sigma
LPS	Sigma
PBS	Sigma
Penicillin/Streptomycin	Gibco
Percoll	Pharmacia
PGE_2	Cayman Chemicals
RPMI 1640	Gibco
Sagatal	May & Baker
superoxide dismutase	Sigma
trypsin	Gibco

INDEX

CHAPTER 1

INTRODUCTION

Prostaglandin and their receptors - discovery and classification

1.1.- Introduction to prostaglandins

The first observation of prostaglandin activity was by Kurzrok and Lieb in 1930 (Kurzrok & Lieb, 1930). This observation was supported and extended by Goldblatt and von Euler in the late 30's and it was von Euler's mistaken belief that the source of the biological activity present in the semen was the prostate gland, that led to the name "prostaglandin", (Goldblatt, 1933; von Euler, 1936). However, the first prostaglandins (PG) were not successfully purified for another 20 years, and these were PGE_1 and $PGF_{1\alpha}$ (Bergström & Sjövall, 1957).

Currently 11 types designated A-J are known to exist of which PGA_2, PGB_2, and PGC_2 are extraction artefacts (Horton, 1979), and PGG_2 and PGH_2 are unstable intermediates in the biosynthesis of this family of mediators (Hamburg & Samuelsson, 1973). Prostaglandins E and F_a, were so named because they partitioned into ether and phosphate buffer (Swedish fosfat) respectively (Horton, 1979), whereas prostaglandins A and B were so named because they can be produced by treatment of prostaglandin E (PGE) with acid and base respectively, (Bergström, 1967). The other prostaglandins were named to fill in the alphabetic sequence. Thromboxane A_2 (TXA_2) was discovered and named separately from the prostaglandins because it has a six- rather than a five-carbon ring but is derived from the same precursors PGG_2 and PGH_2 (Needleman *et al.*, 1976). The collective term for prostaglandins and thromboxanes is prostanoids.

Prostanoids may be regarded as substituted derivatives of prostanoic acid and are classified according to their substitution pattern on the cyclopentane ring. They can be biosynthesised from three related fatty acid precursors, 8,11,14-eicosatrienoic acid (dihomo-γ-linolenic acid), 5,8,11,14-eicosatetranoic acid (arachidonic acid (AA)), and 5,8,11,14,17-eicosapentanoic acid (timodonic acid) generating monoenoic, dienoic and trienoic PGs (Bergström *et al.*, 1964; Gryglewski *et al.*, 1979; Knapp *et al.*, 1978; Needleman *et al.*, 1979). These are better known as 1-, 2- and 3-series prostaglandins of which dienoic, or 2-series, prostanoids whose precursor is AA are the most abundant prostanoids formed by human and animal tissues

(Bergstrom *et al.*, 1964; Needleman *et al.*, 1979). Leukotrienes, lipoxins, and hydroxyeicosatetraenoic acids (HETEs) are also formed from AA, and together with the prostanoids constitute the eicosanoids.

AA is incorporated in phospholipids in an esterified form (Billis *et al.*, 1975). It is generally located at the 2-position in phospholipids and must first be released before enzymatic attack can occur; three pathways have been proposed. Firstly that phospholipase A_2 (PLA_2) catalyses the cleavage of AA from the 2-position (Flower & Blackwell, 1976; Vogt, 1978). This has been reported as the major pathway in the prostanoid cascade of platelets (Broekman, 1986; Derksen & Cohen, 1975; Mahadevappa & Holub, 1986; Purdon *et al.*, 1987). However, others believe there is insufficient PLA_2 activity to account for the burst of AA release that occurs after platelet stimulation (Bell *et al.*, 1979). They do however detect enough diglyceride lipase activity and propose that the PLC pathway is responsible by first forming diacylglycerol (DAG) from which diglyceride lipase releases AA. Another study (Billah *et al.*, 1980) suggests the involvement of both PLC and PLA_2 sequentially, with PLC producing phosphatidic acid from phosphatidyl inositol and PLA_2 subsequently releasing AA.

Irrespective of the phospholipase involved, free AA is converted to prostanoids by a series of oxidation steps. The first step is a double oxidation or cyclo-oxygenase reaction (bis-dioxygenation: Ogino *et al.*, 1978), combining two oxygen molecules on the polyunsaturated precursor. A cyclic molecule is produced exhibiting both 9, 11 endoperoxide and 15-hydroperoxyl groups and called endoperoxide G or PGG_2 (Samuelsson, 1972). All the natural prostaglandins are synthesised from this precursor, see Figure 1.1.

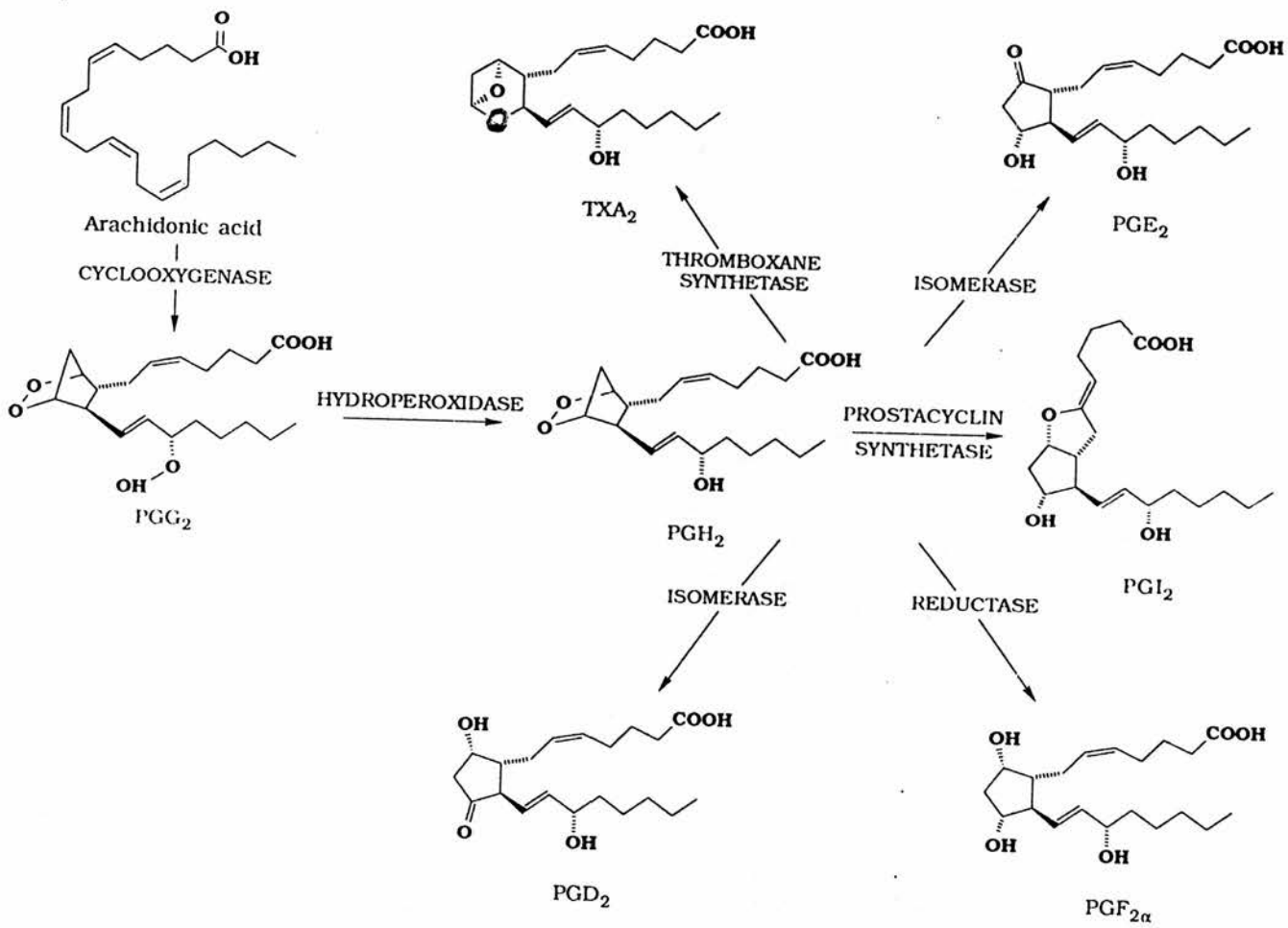


Figure 1.1 The biosynthesis of prostanoids

1.2.- Receptors

There are five primary active AA metabolites:- PGD_2, PGE_2, $PGF_{2\alpha}$, PGI_2 and TXA_2. Five major receptor-types that mediate the actions of these prostanoids have been proposed and named DP, EP, FP, IP and TP respectively (Coleman *et al.*, 1984, 1990). Receptor classification is based on the principal that each natural prostaglandin is more potent at its own receptor by at least one order of magnitude than the other natural prostaglandins.

This thesis is particularly concerned with EP receptors which have a wide distribution in terms of both the tissues and species in which they have been identified, and in the responses which they mediate. The EP receptor has been further subdivided into four subtypes termed EP_1, EP_2, EP_3 and EP_4 (Coleman *et al.*, 1994), and as such, is currently the only prostanoid receptor to have defined subtypes. There is some controversy as regards the TP receptor with certain groups believing the existence of subtypes (Morinelli *et al.*, 1989), however, these results may reflect mRNA splice variants of the TP receptor (Raychowdhury *et al.*, 1994). Since this thesis is concerned with EP-receptor identification and characterisation, only original classification of these subtypes will be discussed.

It was first suggested that two subtypes of PGE_2 receptor may exist by Bennett & Posner (Bennett & Posner, 1971). They found that SC-19220 and polyphloretin phosphate (PPP) blocked the contractile action of PGE_2 on the longitudinal muscle of the guinea-pig ileum whereas the relaxant action of PGE on the circular muscle was unaffected. Since then SC-19220 has been shown to inhibit PGE_2 in only some of a range of EP-receptor containing preparations, such as the guinea-pig ileum and fundus, but not the guinea-pig lung, dog saphenous vein, rabbit aorta, dog and cat iris or cat trachea. EP-receptors blocked by SC-19220 have been classified as EP_1-receptors.

This subclassification of the EP-receptor has been confirmed and extended by the identification of the selective agonists, sulprostone and AY23626. These two compounds showed a different activity profile on

tissues insensitive to SC-19220 or AH6809, another EP_1-receptor antagonist (Coleman *et al.*, 1985), which lead to the division of non-EP_1 receptors into two further subtypes, EP_2 and EP_3 (Coleman *et al.*, 1987a,b,c). Sulprostone has a high potency at EP_1- and EP_3- (chick ileum) receptors, and is inactive at EP_2-receptors (cat trachea), whereas AY23626 has high potency at EP_2- and EP_3-receptors, having weak or no activity at EP_1-receptors.

From these data, EP-receptors were originally classified into three subtypes EP_1, EP_2, and EP_3 (Coleman *et al.*, 1990). However, work on hamster uterus (Coleman 1983; Yeardley *et al.*, 1992a), rabbit jugular vein (RJV) (Lawrence & Jones, 1992) and rat trachea (Lydford & McKechnie, 1993) all provided evidence for the existence of a fourth subtype of EP-receptor. This has now been confirmed by the discovery of a receptor antagonist for this subtype (AH23848B (Louttit *et al.*, 1992a; Coleman *et al.*, 1994, 1995; Milne *et al.*, 1995)) and work on the pig saphenous vein (PSV) an EP-receptor containing preparation with a non-EP_1, -EP_2 or -EP_3 subtype profile (Louttit *et al.*, 1992b).

The effects associated with EP_1- and EP_3-receptors may be considered as excitatory, and are believed to be mediated by a stimulation of phosphatidylinositol turnover and/or inhibition of adenylate cyclase (AC) activity, with a resulting decrease in intracellular cAMP (Gutman *et al.*, 1979; Namba *et al.*, 1993; Strong *et al.*, 1992; Sugimoto *et al.*, 1992a). In contrast, the effects associated with EP_2- and EP_4-receptors may be considered as inhibitory, and are believed to be associated with a stimulation of AC and an increase in intracellular cAMP (Coleman *et al.*, 1994, 1995; Creese & Denborough, 1981; Honda *et al.*, 1993; Jumblatt & Paterson, 1991; Milne *et al.*, 1995; Reimer *et al.*, 1992; Yeardley *et al.*, 1993).

The constant problem throughout the classification of prostaglandin receptors has been the lack of selective antagonists, see Table 1.1. TXA_2 is a potent inducer of platelet aggregation and contraction of vascular and respiratory smooth muscle (Moncada *et al.*, 1978) and the development of antagonists at this receptor has yielded many potent compounds such as GR32191X which has a pA_2 of 8.2-8.8

(Lumley *et al.*, 1989) and EP092 with a pA_2 of 7.2-8.4 (Armstrong *et al.*, 1985).

Interestingly, very few antagonists have been discovered for the PG receptor subtypes and may reflect the fact that most analogues have been based on the structure of PGs, Figures 1.2-1.5. It seems that basing structures on PGs produces agonists rather than antagonists and that to develop antagonists, compounds have needed to be distinctly different from PGs, such as AH6809, Figure 1.5,. This has made the discovery of antagonists difficult because there are no indications as to what structural confirmation will allow a non-prostaglandin like compound to bind to a receptor but exhibit no agonism.

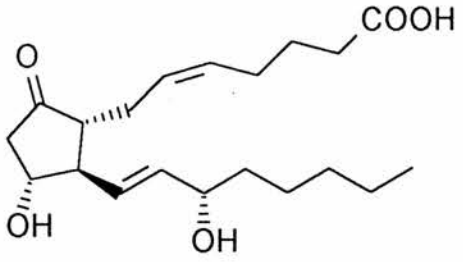
Nevertheless, there are now selective agonists for almost every prostanoid receptor plus a few selective antagonists and this, coupled with preparations containing specific receptor populations, has allowed the development of a working classification for the prostanoid receptors (Coleman *et al.*, 1984, 1990) as shown in Table 1.1.

There are currently several PG analogues being marketed or undergoing clinical trials as abortifacients (gemeprost, misoprostol & sulprostone), antiulcer (misoprostol), antihypertensive (limaprost; tiprostanide), or antiglaucoma agents (latanoprost) (Collins & Djuric, 1993).

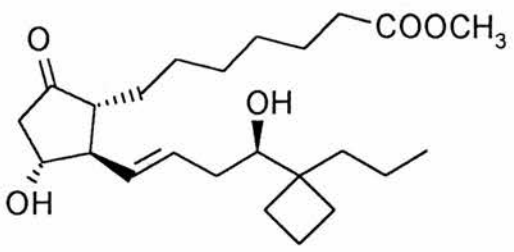
Receptor	Natural prostaglandin	Synthetic agonists		Synthetic antagonists	
DP	PGD_2	BW 245C ZK110841	(Town *et al.*, 1983) (Ito *et al.*, 1990)	BW A868C AH6809†	(Giles *et al.*, 1989) (Keery & Lumley, 1988)
FP	$PGF_{2\alpha}$	fluprostenol cloprostenol	(Dong & Jones, 1982) (Coleman, 1983)	n.a.	
IP	PGI_2	iloprost cicaprost	(Schror *et al.*, 1981) (Sturzebecher *et al.*, 1985)	n.a.	
TP	TxA_2	U46619 EP171	(Malmsten, 1976) (Wilson & Jones, 1985)	GR32191 EP092 AH23848‡	(Lumley *et al.*, 1989) (Armstrong *et al.*, 1985) (Brittain *et al.*, 1985)
EP_1	PGE_2	sulprostone*	(Coleman *et al.*, 1987a)	AH6809† SC-19220	(Coleman *et al.*, 1985) (Coleman *et al.*, 1985)
EP_2	PGE_2	butaprost AH13205	(Gardiner, 1986) (Nials *et al.*, 1993)	AH6809†	(Woodward *et al.*, 1995)
EP_3	PGE_2	misoprostol GR63799X sulprostone*	(Reeves *et al.*, 1988) (Bunce *et al.*, 1990) (Coleman *et al.*, 1987a)	n.a.	
EP_4	PGE_2	n.a.		AH23848‡ AH22921	(Louttit *et al.*, 1992b) (Coleman *et al.*, 1995)

Table 1.1 - Selective agonists and antagonists at prostanoid receptors

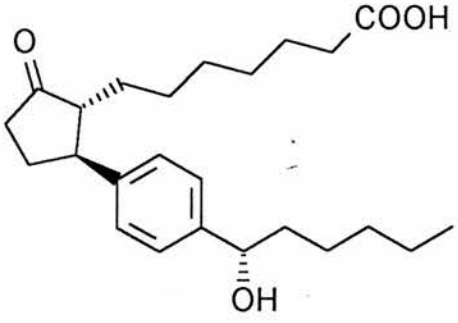
* EP_1 and EP_3 agonist
† DP, EP_1, EP_2 and TP antagonist
‡ TP and EP_4 antagonist
n.a.- not any



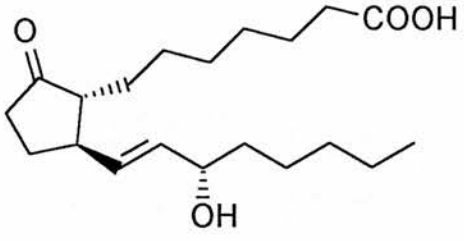
PGE_2



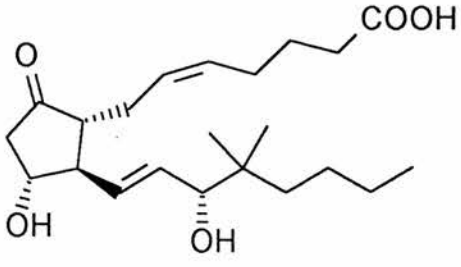
Butaprost



AH 13205

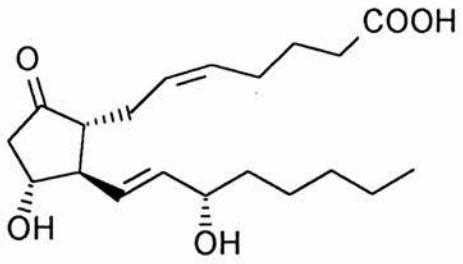


11-deoxy PGE_1

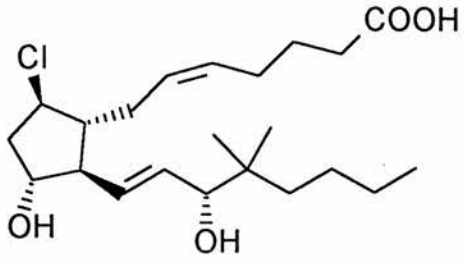


16,16-dimethyl PGE_2

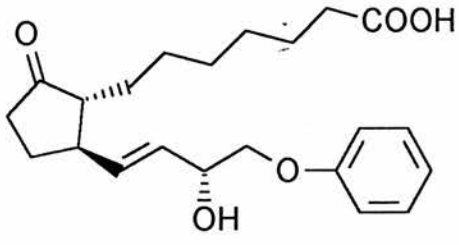
Figure 1.2: Structure of PGE analogues



PGE_2



Nocloprost



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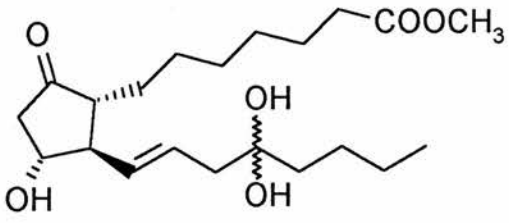
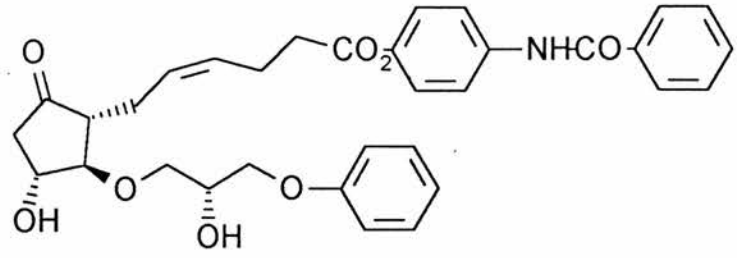
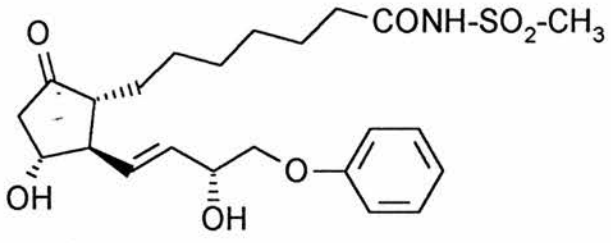


Misoprostol

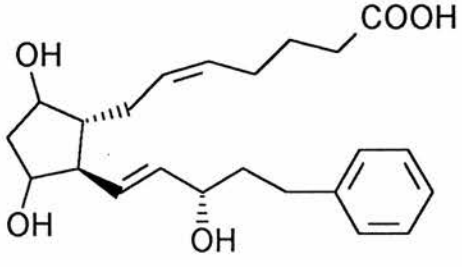
Figure 1.3: Structure of PGE analogues



GR 63799X

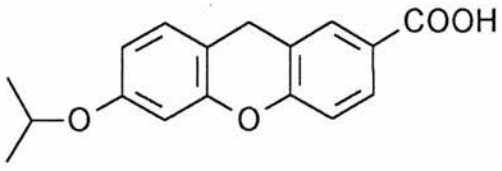


Sulprostone

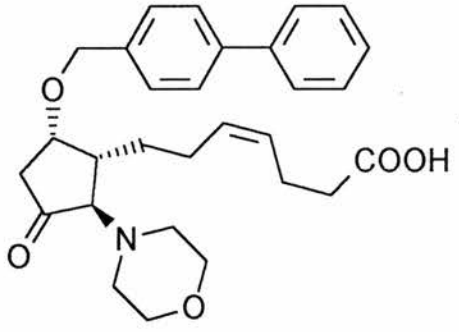


17-phenyl-ω-trinor $PGF_{2\alpha}$

Figure 1.4: Structure of PG analogues



AH 6809



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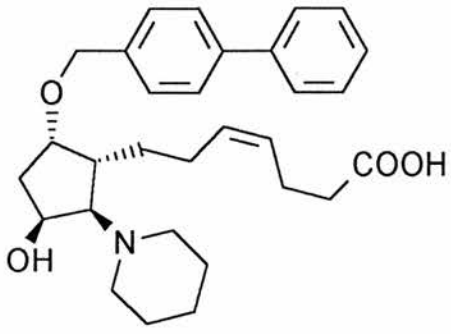

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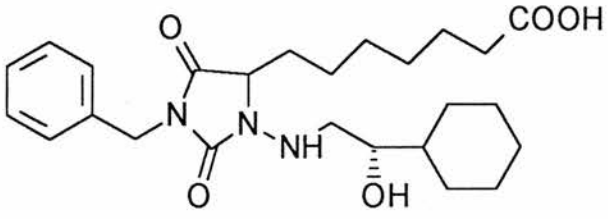

BW A868C

Figure 1.5: Structure of PG antagonists

EP_1 receptors

With regards to the EP receptors, there are now many selective agonists and some antagonists, see Table 1.1 and 1.2. Sulprostone is equipotent with PGE_2 at the EP_1 receptor (Coleman *et al.*, 1987a) but has also been shown to stimulate EP_3 receptors, and although sulprostone was first identified as an EP_1 agonist, it is actually a more potent agonist at EP_3 receptors, Table 1.2 (Coleman *et al.*, 1987c). 17-phenyl-ω-trinor PGE_2 is an EP_1 agonist but with limited selectivity having only a 10-fold greater potency at the EP_1 receptor than EP_2 and EP_3 receptors (Lawrence *et al.*, 1992). There are a few antagonists for EP_1 receptors. SC-19220 was the first identified EP_1 receptor antagonist (Coleman *et al.*, 1985, 1987a) followed by AH6809, a more potent EP_1 antagonist (Coleman *et al.*, 1985, 1987a). AH6809, however, has also been shown to have DP antagonist activity (Keery & Lumley, 1988) and, most recently, it has been reported to also have antagonist activity at the recombinant human EP_2 receptor (Woodward *et al.*, 1995).

EP_2 receptors

EP_2 agonists have helped in the initial classification of EP receptor subtypes as described above. AY23626 is equieffective with PGE_2 in the cat trachea, Table 1.2 (Coleman *et al.*, 1987b), but is also an agonist at EP_3 receptors (Coleman *et al.*; 1987a,b). Butaprost (first published as TR4979) and AH13205 are reported to be selective EP_2 agonists (Gardiner, 1986; Nials *et al.*, 1993) but are both less potent compounds than PGE_2 at the EP_2 receptor, see Table 1.2. AH6809 is the only reported compound with antagonist activity at EP_2 receptors (Woodward *et al.*, 1995; personal communication Prof. R. L. Jones & Dr. R. A. Coleman).

AGONIST				ANTAGONIST			
Receptor subtype	**Compound**	**EEC**	**Reference**	**Receptor subtype**	**Compound**	**pA_2**	**Reference**
EP_1	sulprostone	4-6	Coleman *et al.*, 1987a	**EP_1**	SC-19220	5.2-5.6	Bennett and Posner, 1971 Coleman *et al.*, 1985, 1987a,b
	17-phenyl-ω-trinor PGE_2	1	Lawrence *et al.*, 1992		AH6809	6.4-7.0	Coleman *et al.*, 1985, 1987a
EP_2	butaprost	6-30	Gardiner, 1986	**EP_2**	AH6809	150†	Woodward *et al.*, 1995
	AH13205	30-100	Nials *et al.*, 1993				
	AY23626	2-14	Coleman *et al.*, 1987a,b				
	misoprostol	1-4	Humbles *et al.*, 1991				
EP_3	GR63799X	0.1	Bunce *et al.*, 1990	**EP_3**	n.a.		
	misoprostol	0.2-1.0	Reeves *et al.*, 1988 Bunce *et al.*, 1990				
	sulprostone	0.05-0.3	Coleman *et al.*, 1987b Lawrence *et al.*, 1992				
	M&B28767	0.6	Lawrence *et al.*, 1992				
EP_4	n.a.			**EP_4**	AH23848	5.3-6.2	Coleman *et al.*, 1994, 1995 Milne *et al.*, 1994, 1995
					AH22921	5.3	Coleman *et al.*, 1994, 1995

Table 1.2 - Agonist and antagonist potencies at the EP receptors for a range of selective compounds

EEC equieffective concentration
n.a. not any
†- estimate of K_i using K_D for PGE_2 of 26 nM (Regan *et al.*, 1994)

EP_3 receptors

The development of EP_3 agonists has been helped by the market for potential antiulcer drugs (Collins, 1986). Misoprostol was developed as such a compound and is equieffective with PGE_2 at EP_3 receptors. However, it also has potent EP_2 agonist activity (Reeves *et al.*, 1988; Bunce *et al.*, 1990; Lawrence *et al.*, 1992), Table 1.2. Sulprostone has been shown to have potent agonist activity at EP_3 receptors (Lawrence *et al.*, 1992), but again is limited by its lack of selectivity, also being a potent EP_1 agonist (Coleman *et al.*, 1987a). GR63799X is reported as being a EP_3 agonist and is more potent at the EP_3 receptor than PGE_2 with an equieffective concentration (EEC) of 0.1 (Bunce *et al.*, 1990). GR63799X has been tested in vivo in rats for its inhibition of lesion formation and was as effective as misoprostol. Furthermore, it produced fewer side-effects of uterine stimulation. However, like sulprostone, GR63799X also has agonist activity at EP_1 receptors (Bunce *et al.*, 1990).

EP_4 receptors

As mentioned earlier, EP_4 receptors have been the most recent EP receptor subtype to be identified and were first described in the PSV using the antagonist AH23848B (Louttit *et al.*, 1992a). To date there are no published data on EP_4 agonists, but there are two published antagonists AH22921 and AH23848B (Coleman *et al.*, 1994, 1995). These two compounds are both weak antagonists with pA_2s of 5.3 and 5.3-6.2 respectively, see Table 1.2.

With the available agonists and antagonists it is now possible to identify which receptor subtypes are present in tissues by functional profile. However, this information has been superseded by the cloning of all prostanoid receptors currently identified and the availability of selective probes (Pierce *et al.*, 1995).

1.3.- Receptor cloning

The purification of a TP receptor from human platelets was the first step in the cloning of prostanoid receptors. From the partial amino acid sequence, an oligonucleotide probe was designed and a cDNA encoding a full length TP receptor cloned from a human placenta library (Hirata *et al.*, 1991). The deduced amino acid sequence of this receptor consisted of 343 amino acids and showed structural similarity with other previously cloned G-protein-coupled receptors. Homology based studies rapidly led to the cloning of cDNAs encoding the human DP (Boie *et al.*, 1995), FP (Abramovitz *et al.*, 1994), IP (Boie *et al.*, 1994) and four subtypes of the EP receptor; EP_1, EP_2, EP_3 and EP_4 (reviewed in Pierce *et al.*, 1995).

From their predicted secondary structure, the cloned prostanoid receptors fit the general model for the G-protein-coupled receptors like the rhodopsin-type receptors (Savarese & Fraser, 1992) in which there are seven putative transmembrane domains, an extracellular amino terminus and an intracellular carboxyl terminus. Functional expression of the cloned receptors is also consistent with a single subunit structure, which contains both the ligand binding and second messenger coupling site.

Like the other subfamilies of the G protein-coupled receptors (e.g. adrenoceptors and 5-HT receptors), the prostanoid receptors do not share extensive overall amino acid identity. Comparison of all members of the human prostanoid receptor family shows 28% to 45% amino acid sequence homology between any two members (Boie *et al.*, 1995). There are a total of 28 amino acid residues conserved in all prostanoid receptors, 8 of which are also conserved in all rhodopsin-like G-protein-coupled receptors.

Two regions are particularly well conserved in all the prostanoid receptors, see Table 1.3. One of these is in the second extracellular loop, connecting the fourth transmembrane domain with the fifth transmembrane domain, and contains the non-variable and unique tripeptide WCF. The cysteine residue within the tripeptide forms a

putative disulfide bridge with the conserved cysteine residue in the first extracellular loop (Boie *et al.*, 1995). In this same sequence there is a seven residue motif, PGTWCFI, conserved among all the prostanoid receptors that bind PGE_2 suggesting a role in specificity, see Table 1.3.

The second region in the seventh transmembrane domain contains approximately 15 residues that are highly conserved, including five non-variable residues. The non-variable Arg in the seventh transmembrane domain corresponds to the Lys residue in rhodopsin, which is essential for retinal binding, suggesting the possibility that the Arg provides a binding site for the α-carboxylic acid group of the prostanoids (Hirata *et al.*, 1991). There is also another invariant tripeptide, DPW, which includes the proline residue conserved in all G-protein-coupled receptors (Probst *et al.*, 1992).

For all the cloned prostanoid receptors, except the EP receptors, there has been a good correlation between the pharmacology of each heterologously expressed receptor and the pharmacology as defined in native tissues. The EP receptors, however, did not show a good correlation. A mouse clone (Honda *et al.*, 1993) initially designated as the EP_2 receptor was found to be inactive with respect to the binding of butaprost, a previously characterised selective EP_2 receptor agonist. The recombinant human homologue (Bastien *et al.*, 1994), originally designated as an EP_2 receptor, also failed to bind butaprost. The subsequent cloning of a fourth subtype of EP receptor, for which butaprost had affinity, suggested that it was the human EP_2 receptor (Regan *et al.*, 1994a), and that these previously cloned 'EP_2' receptors represented a different subtype. It has recently been reported that the previously cloned 'EP_2' receptors (Bastien *et al.*, 1994; Honda *et al.*, 1993) are probably EP_4 receptors. Comparing the primary sequences of the cloned EP_2 and EP_4 receptors shows that they only share approximately 40% amino acid identity in their transmembrane domains and are likely to represent the products of separate genes (Pierce *et al.*, 1995).

Receptor subtype	Second extracellular loop	Seventh transmembrane domain
EP_2	PGT**WCF**I	**R**FLSINS**I**I**DPW**VFA
EP_4	PGT**WCF**I	**R**IASVNP**I**L**DPW**IYI
EP_1	PGT**WCF**I	**R**LASWNQ**I**L**DPW**VYI
EP_3	PGT**WCF**I	**R**LASLNQ**I**L**DPW**VYL
DP	PGT**WCF**I	**R**FLSVIS**I**V**DPW**IFI
TP	PGS**WCF**L	**R**VATWNQ**I**L**DPW**VYI
IP	PGS**WCF**L	**R**FYAFNP**I**L**DPW**VFI
FP	SRT**WCF**Y	**R**MATWNQ**I**L**DPW**VYI

Table 1.3 - Sequence homologies of two regions in the cloned human prostanoid receptors.

Non-variable residues shown in bold
Source - Boie *et al.*, 1995

Phylogenetic analyses (Boie *et al.*, 1995; Pierce *et al.*, 1995), show two major branches of the prostanoid receptors with one group (IP, DP, EP_2 and EP_4 receptors) activating AC and the other group either stimulating phosphatidylinositol hydrolysis (TP, FP and EP_1 receptors) or inhibiting AC (EP_3 receptors).

Of interest in the EP subtypes is the presence of multiple EP_3 isoforms, i.e. mouse $EP_{3\alpha}$, $EP_{3\beta}$ and $EP_{3\gamma}$, and bovine EP_{3A}, EP_{3B}, EP_{3C} and EP_{3D}, which differ only in their carboxyl-terminal tails (Sugimoto *et al.*, 1992b; Namba *et al.*, 1993; Irie *et al.*, 1993). Furthermore, rabbit and rat EP_3 subtypes also have multiple isoforms with divergent carboxyl-terminal tails (Takeuchi *et al.*, 1993; Breyer *et al.*, 1994). In humans, seven different cDNAs have been cloned that can give rise to different isoforms (Adam *et al.*, 1994; An *et al.*, 1994; Regan *et al.*, 1994b; Kotani *et al.*, 1995; Schmid *et al.*, 1995). Structurally, this extensive alternative splicing of the EP_3 receptor only affects the carboxyl terminus and all the isoforms have the same amino termini and transmembrane domains and only begin to differ eleven residues in the direction of the carboxyl terminus from transmembrane seven (Pierce *et al.*, 1995). Since phosphorylation of the carboxyl terminus is involved in the desensitisation of G-protein-coupled receptors (Lefkowitz, 1993), these splice variants may explain differences in desensitisation seen between the mouse (Negishi *et al.*, 1993a) and human (An *et al.*, 1994) EP_3 isoforms. Another way in which the EP_3 receptor isoforms may functionally differ from one another is in their coupling to second messenger pathways. It has been suggested that the carboxyl terminus may help determine the specificity of G-protein coupling (Namba *et al.*, 1993). Kotani and co-workers even suggest that certain effects of PGE_2 on smooth muscle relaxation, which are believed to be mediated by EP_2 receptors, may be partially due to two isoforms $EP_{3\text{-II}}$ and $EP_{3\text{-IV}}$ which, when transfected into COS-7 cells, generate increases in intracellular cAMP in the presence of the EP_3 agonist M&B28767 (Kotani *et al.*, 1995).

High receptor expression and choice of cell type may, however, be the reason these splice variants couple to multiple second messenger pathways. The G-protein content of cells such as the CHO cell and

COS-7 cell may be different from that found *in vivo* for the tissues which express these subtypes. If a receptor has high affinity for G_i over G_s *in vivo* but is expressed in a cell line containing mainly G_s, then high receptor expression could favour G_s activation and any subsequent measurement of intracellular changes would not reflect the true nature of the receptor.

1.4.- Background

At the start of my thesis, January 1993, the EP receptors were classified into three subtypes, EP_1, EP_2 and EP_3 (Coleman *et al.*, 1990). Certain publications, however, indicated the existence of another EP receptor subtype (Lawrence & Jones, 1992; Yeardley *et al.*, 1992a). These receptors generate relaxation in the presence of PGE_2 but give much higher EEC ratios for certain EP_2 receptor agonists when compared to PGE_2. For example, Lawrence and Jones (1992), showed butaprost to have an EEC ratio of 685 against PGE_2 in the RJV which is about 40 times greater than the EEC of 17 for butaprost against PGE_2 in the cat trachea, an accepted EP_2 receptor containing preparation (Gardiner, 1986). Yeardley and co-workers were investigating the EP receptor subtype involved in the inhibition of uterine activity (Yeardley *et al.*, 1992a). The group found that PGE_2, but not sulprostone or AH13205, inhibited electrically-induced uterine contractions and caused concentration-related increases in cAMP levels in hamster uterus. They concluded that the high potency of PGE_2 supported the involvement of EP receptors, but the lack of activity of sulprostone and AH13205 cast doubt on the involvement of EP_1, EP_2 or EP_3 receptor subtypes.

The differences in these particular systems which suggested the involvement of a second relaxant EP receptor subtype were only just beginning to be explained in January 1993. Two abstracts published at the 8th International Conference on Prostaglandins and Related Compounds in Montreal by James Louttit (Louttit *et al.*, 1992a,b) demonstrated that the PSV contained an EP receptor which did not appear to be of the EP_1, EP_2 or EP_3 subtypes (Louttit *et al.*, 1992a). They also demonstrated selective antagonist activity for AH23848B at the EP receptor subtype present on the PSV (Louttit *et al.*, 1992b) and concluded the presence of a novel EP receptor subtype and suggested naming it the EP_4 receptor.

Our group's interest in the field at the time concerned the RJV. It had previously been described as an 'atypical EP_2' receptor containing preparation and we wished to further investigate this classification.

Allergan Inc., USA, were funding the PhD thesis and at the time were attempting to develop antiglaucoma agents based on the knowledge that $PGF_{2\alpha}$ lowers intraocular pressure (IOP) (Alm & Villumsen, 1989). $PGF_{2\alpha}$, however, also causes the undesirable side effect of hyperaemia, redness around the white of the eye (Protzman & Woodward, 1990). David Woodward and co-workers had discovered that compounds which induced hyperaemia also relaxed the RJV and that this preparation was a useful negative screen. However, the mechanism by which $PGF_{2\alpha}$ mediated these responses had not been elucidated.

The Chinese hamster ovary (CHO) cell line had been discovered to contain an EP receptor linked to AC which was sensitive to block by AH23848B (Dr. R. A. Coleman personal communication), and our group decided that this could prove a useful preparation to collect more data about the EP_4 receptor, if this was indeed the receptor expressed in these cells.

Several studies on monocyte/macrophages have shown that PGE_2 is capable of acting as an endogenous negative feedback control on physiological responses such as locomotion, phagocytosis and colony formation (Pelus *et al.*, 1979; Oropeza-Rendon *et al.*, 1979, 1980; Bonney *et al.*, 1980). However, little work has been carried out to determine which subtype(s) of EP receptor are present, and whether these are directly linked to the physiological functions observed with PGE_2. Binding studies have been carried out on human monocyte membranes (Eriksen *et al.*, 1985) but using only PGE_2 and other natural prostanoids as displacing agents. Misoprostol has been investigated along with PGE_2 (Haynes *et al.*, 1992; Reder *et al.*, 1994) but only from the objective of inhibiting whole animal disease onset and growth as well as cytokine release by mouse and human monocyte/macrophages. As yet, the inhibitory EP receptors present on human monocytes has not been identified with respect to the currently known subtypes.

1.5.- Aims

The aim of this thesis was twofold. Initial studies involved the confirmation and further characterisation of this newly described EP_4 receptor subtype. For this purpose, a range of agonists and, where possible, antagonists were investigated using the CHO cells and the PSV and RJV preparations. The strategy being to build up information on the EP_4 receptor subtype.

The second and main aim of this thesis was to classify the EP receptor subtype present in human monocytes. As described previously, PGE_2 has been shown to have inhibitory effects on human monocyte/macrophages, and these effects are likely to be mediated via EP_2 and/or EP_4 receptors. As little information had been published with respect to EP_4 receptors, our aim was to use data on EP_4 receptors obtained in the first part of this thesis along with already published data at EP_2 receptors, to classify the inhibitory EP receptor(s) expressed on human monocytes.

The EP receptors present on human monocytes was studied to see if they were directly coupled to AC, and how different EP agonists and the antagonist AH23848B affected the resultant accumulation of cAMP. The whole cell system and the functions of PGE_2 on the human monocyte were then investigated. PGE_2 was studied from the standpoint that it is an inhibitor of certain monocytic cell functions, and thus only inhibition of stimulatory systems by PGE_2 were investigated. It is well documented that PGE_2 also possesses stimulatory and pro-inflammatory actions, but the objective of the second part of this thesis was to identify if there was a role for selective EP agonists targeted at the human monocyte. Studies on the effects of PGE_2, and the receptor mediating the response, were carried out on IL-1α and IL-1β release and superoxide anion generation from the human monocyte. The second messenger system mediating the inhibitory effects of PGE_2 against superoxide anion generation was also investigated.

CHAPTER 2

Investigation of the EP-receptor subtype(s) expressed by the Chinese Hamster Ovary cell line, and further investigation of the relaxant EP-receptor(s) present on the Pig Saphenous Vein and Rabbit Jugular Vein

CHAPTER 2.1

Investigation of the EP-receptor subtype(s) expressed in the Chinese Hamster Ovary cell line

2.1.1.- Introduction

The Chinese Hamster Ovary (CHO) cell line was first developed by Tjio and Puck in 1957 (Tjio & Puck, 1957). The uses of the CHO cell line have been diverse since its discovery, but it is mainly used nowadays for expression of transfected cloned sequences. Cells are generally grown in α minimal essential medium or Hams F12 medium supplemented with 5-10% foetal calf serum and antibiotics/fungicides (amounts of antibiotics/fungicides ranged between publications as well as the types used).

Literature on transfected CHO cells show a diverse field both in terms of systems studied and species involved, i.e. rat dopamine D_2 receptor (Kanterman *et al.*, 1990), human D_4 receptors (Asghari *et al.*, 1995), mouse T-cell IL-1 receptor (Curtis *et al.*, 1989) human lipoxin A_4 receptor (Fiore *et al.*, 1994) and almost all the prostanoid receptors from a variety of species (Sugimoto *et al.* 1992; Watabe *et al.*, 1993; Hirata *et al.*, 1994; Ito *et al.*, 1994; Namba *et al.*, 1994; Katsuyama *et al.*, 1995).

Prostaglandin receptors linked to activation of AC have been transfected in CHO cells. Mouse DP and EP_2 receptors were cloned and transfected into CHO cells which have been used for cAMP assays and binding analysis to verify functional characteristics of the expressed receptors (Hirata *et al.*, 1994; Katsuyama *et al.*, 1995). The mouse IP receptor has also been cloned and transfected into CHO cells allowing cAMP measurement and [^{3}H]iloprost binding studies which confirmed stable expression of the IP receptor (Namba *et al.*, 1994).

Receptors which stimulate phosphatidylinositol have also been transfected and expressed in CHO cells. Bovine FP receptors have been shown to elevate $[Ca^{2+}]_i$ and purified $G_{q\alpha}$ antibody was shown to inhibit receptor activation (Ito *et al.*, 1994), thus linking FP receptor activation with G_q coupling. With mouse recombinant EP_1 receptors expressed in CHO cells, 17-phenyl-ω-trinor PGE_1, sulprostone and PGE_2 were equipotent at displacing bound [^{3}H]PGE_2 from crude membrane fractions confirming the expression of EP_1 receptors

(Watabe *et al.*, 1993). Signal transduction in the cloned EP_1 receptor was later studied in these EP_1 receptor expressing cells and shown to be mediated by PKC, and not PKA or PLC, activation (Katoh *et al.*, 1995)

The CHO cell line has been used extensively to study EP_3 receptor splice variants and their G-protein coupling. The mouse EP_3 receptor has been cloned and expressed in CHO cells (Sugimoto *et al.* 1992) and later identified as expressing different isoforms, $EP_{3\alpha}$ and $EP_{3\beta}$, by alternative splicing of the EP_3 gene (Sugimoto *et al.*, 1993). These two isoforms were shown to have the same ligand binding properties in transfected COS-1 cell membranes, but different profiles for inhibition of forskolin induced cAMP generation and stimulation of GTPase activity in transfected CHO cells (Sugimoto *et al.*, 1993). Further work with the same transfected CHO cell line showed different desensitisation profiles to PGE_2 for these two subtypes (Negishi *et al.*, 1993). These two isoforms expressed in CHO cells have also been shown to enhance AC activity after initially sensitising the cells with PGE_2 (Harazono *et al.*, 1994), again showing different profiles and highlighting the capacity of DNA splicing to generate functionally different end-products with identical binding capacity.

A third isoform of mouse EP_3, $EP_{3\gamma}$, was identified, and expressed in CHO cells, and shown to both stimulate and inhibit AC (Irie *et al.*, 1993). In CHO cells expressing these three isoforms of mouse EP_3 receptors, PGE_2 increased $[Ca^{2+}]_i$ and accumulation of IP_3 (Irie *et al.*, 1994). This effect was sensitive to block by pertussis toxin and a PLC inhibitor U-73122. The group concluded that these EP_3 isoforms are linked to PLC activation via G_i, and that PLC activation leads to Ca^{2+} mobilisation.

Namba and co-workers studied further the G-protein specificity of EP_3 receptors in CHO cells with four splice variants from bovine adrenal medulla (Namba *et al.*, 1993). The group found that three of the splice variants EP_{3B}-EP_{3D} increased cAMP after stimulation with M&B28767 in transfected CHO cells, which was inhibited by a specific anti-$G_{s\alpha}$ antibody, and that two isoforms EP_{3A} and EP_{3D} inhibited forskolin induced cAMP generation, which was potentiated by pertussis toxin.

With further studies on IP_3 generation and $[Ca^{2+}]_i$ measurement, the group concluded that EP_{3A} receptors couple to G_i/G_o protein, EP_{3B} and EP_{3C} couple to G_s, and EP_{3D} couple to G_i, G_s and G_q (Namba *et al.*, 1993). Interestingly, using CHO cell membranes transfected with EP_{3C} receptors, Negishi and co-workers have demonstrated that these receptors stimulate the GTPase activity of G_s, while they inhibit that of G_o by increasing the binding affinity of G_o for GDP (Negishi *et al.*, 1993b) showing further diversities for G-protein coupling and second messenger systems of EP receptor splice variants

Human isoforms of the EP_3 receptor have also been identified and expressed in CHO cells (Kotani *et al.*, 1995). Currently four isoforms have been identified, $EP_{3\text{-I}}$, $EP_{3\text{-II}}$, $EP_{3\text{-III}}$ and $EP_{3\text{-IV}}$, which, like the bovine isoforms have similar affinities to PGE_2 with divergent downstream signalling pathways. M&B28767 increased cAMP in cells expressing $EP_{3\text{-II}}$ and $EP_{3\text{-IV}}$, whereas it inhibited forskolin induced cAMP accumulation in cells expressing all EP_3 isoforms. M&B28767 also stimulated phosphoinositide turnover in cells expressing $EP_{3\text{-I}}$ and $EP_{3\text{-II}}$ showing similar variability with the human isoforms as for the bovine splice variants (Namba *et al.*, 1993).

The usefulness of the CHO cell is highlighted by the number of different receptors transfected into them, and the number of diverse studies carried out with these transfected cells. CHO cells transfected with prostanoid receptors have helped immensely in the characterisation of those prostanoid receptors and have even been used as screens for selective compounds. Negishi and co-workers, used CHO cells transfected with IP, EP_1, EP_2 and EP_3 receptors to identify TEI-3356, a potent and highly selective EP_3 agonist, equieffective with sulprostone at the EP_3 receptor but with limited EP_1, EP_2 and IP agonist activity (Negishi *et al.*, 1994). As many tissues express multiple EP receptor subtypes along with other prostanoid receptors, evaluation of the potency of an agonist or antagonist for a single type of receptor is very difficult. Negishi and co-workers conclude that assay systems involving transfected cells allow clearer evaluation of the potency and selectivity of agonists or antagonists at a single receptor.

EP_4 receptors have only been studied in smooth muscle, with studies concentrating mainly on the antagonists selective for this receptor (Louttit *et al.*, 1992a,b; Milne *et al.*, 1995; Coleman *et al.*, 1994,1995). Interestingly, the Chinese hamster ovary (CHO) cell line has been found to express endogenously an EP receptor positively coupled to AC with characteristics of the EP_4 receptor , i.e. sensitivity to block with AH23848B (Dr. R. A. Coleman, personal communication). This is surprising as cells used for transfection are chosen because they lack the receptor to be transfected. As such, binding studies for EP receptors transfected into the CHO cell line should have detected EP receptors on the native cells, but this has not been documented.

EP_2 receptors have been shown to be positively coupled to AC and induce accumulation of cAMP in rat cortical collecting tubule (RCCT) cells (Sonnenburg & Smith, 1988). PGs of the E-series were most potent at stimulating cAMP, and sulprostone failed to stimulate cAMP production, suggesting the involvement of the EP_2 receptor in cAMP generation. In cultured RCCT cells, PGE_2 was shown to bind to a single class of receptors, and this binding was inhibited by guanine nucleotide derivatives, indicating the involvement of a G-protein (Sonnenburg *et al.*, 1990). The CHO cell line thus offered a good opportunity to study receptor stimulation versus second messenger stimulation and to see if the putative EP_4 receptor present on these cells was positively coupled to AC activation.

We have used a range of agonists at EP receptors and the antagonist, AH23848B (Louttit *et al.*, 1992a), to investigate cAMP generation in native CHO cells. These cells were bought from the European Collection of Animal Cell Cultures (ECACC) to be sure that they were homologous with other CHO cell lines and the passage number was always kept below twenty. Initially, a sensitive RIA for acetylated cAMP was set up and with this assay it was possible to measure cAMP from cells at low adherent concentrations avoiding the possible changes present when cell cultures become confluent.

2.1.2.- Methods

Materials

HBSS - Hanks' Balanced Salt Solution without Phenol Red stored at 20°C.
DMSO - Sterile dimethyl sulphoxide stored at 20°C
IBMX - 3-Isobutyl-1-methylxanthine ($C_{10}H_{14}N_4O_2$ - FW=222.2). Stored as dry powder at 20°C
HAMS F-12 - Nutrient Mixture F-12 (HAM) with L-Glutamine. Stored at 4°C.
Pen/Strep - Penicilin-Streptomycin Solution, containing 10000 U/ml Penicillin and 10000 µg/ml Streptomycin. Stored at -20°C.
FCS - Foetal Calf Serum Heat Inactivated. Mycoplasma and virus screened. Stored at -20°C.
Trypsin - Trypsin-EDTA Solution (1X) prepared in modified PUCK's saline A and stored at -20°C.
Cell culture medium - Cell culture medium was made up with HAMS F-12 minimal alpha medium (500 ml), FCS (50 ml) and Pen/Strep (5 ml), and stored at 4°C. The medium was always warmed to 37°C before use.
Drugs - All drugs dilutions were prepared fresh each day in 0.9% saline from dry powder or ethanol stocks. AH23848B solution was sonicated for 30 min prior to use.

Chinese Hamster Ovary cell culture

The Chinese Hamster Ovary (CHO) cell line was obtained from the European Collection of Animal Cell Cultures (ECACC). The vial was left to defrost fully for 2 min at room temperature then placed in a water bath at 37°C for a further 2 min. Warmed cell culture medium (1 ml) was added, and the contents of the vial diluted to 30 ml with medium. Since the cells are stored in DMSO 10%, it is important to dilute out and completely remove DMSO, as this can cause differentiation of some growing cell cultures. The suspension was centrifuged, 450 g for 10 min at room temperature, and the supernatant discarded. The pellet was immediately resuspended in medium (5 ml) and aliquoted into a 800 ml cell culture flask

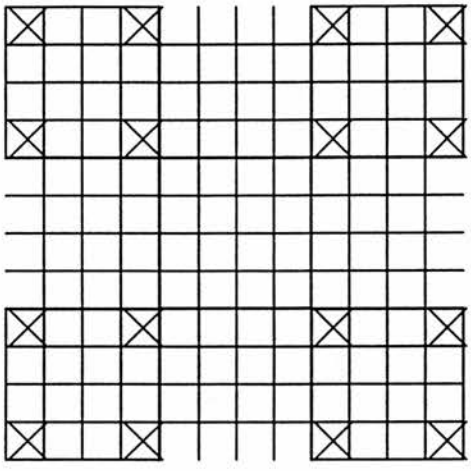

Figure 2.1.1- Haemocytometer slide

The crossed sections were counted to obtain cell count and cell concentration counted as follows:

Total cells counted/4 = Y x 10^4 cells/ml
x5 (dilution factor of cells in Trypan Blue®) = cells/ml in flask
x ? (aliquot volume) = cells in flask

Cyclic AMP extraction from CHO cells

Cells were harvested from their flasks as described above, resuspended in culture medium and seeded to 12 well plates at a concentration of 1 x 10^6 cell/well. The plates were then placed back in the incubator for 1 hr to allow the CHO cells to adhere to the wells.

Once adhered, the medium was removed and the cell monolayer washed twice with 0.9% saline. Drugs were made up fresh each day in HAMS-F12 medium with indomethacin 5 μM and IBMX 0.25 mM, without FCS or Pen/Strep and added immediately in 1 ml aliquots to the cell monolayer. The plates were returned to the incubator and incubated at 37°C. After incubation the medium was removed to tubes and the reaction stopped with ice cold 100% ethanol (1 ml) added directly to the wells or 2 ml to the tubes of medium. The wells were scraped with the rubber tip of a syringe plunger and the ethanol removed. Another 1 ml ethanol 100% was added to each well and again the wells scraped before removing this ethanol. Cell debris was removed from the samples by centrifugation at 450 g for 10 min. The ethanol layer was then decanted to clean, labelled tubes which were evaporated to dryness at 55°C in heating blocks under a stream of air. The dry tubes were covered and stored at -20°C until assayed.

A time course was run initially to determine the optimum incubation time for the CHO cells. In addition samples were taken from both the cellular and medium fractions to determine which samples produced the maximum increase in cAMP levels within a 30 min time course, Figure 2.1.5. From these, it was decided that 5 min was the optimum incubation time, and this was used throughout all the other experiments.

For antagonist studies, antagonists were added to the monolayer, 10 min prior to the agonist, in 0.9 ml aliquots of HAMS F-12 and incubated at 37°C. Agonists were then added in 100 μl aliquots and the plates shaken gently before incubation at 37°C.

2.1.3.- cAMP RIA

Materials

50 mM sodium acetate buffer, pH-6.0 - 0.05 M sodium acetate anhydrous (MW = 82.03) - 4.10 g/l + 0.05 M acetic acid - 2.86 ml glacial acetic acid/l. Sodium acetate buffer was brought to pH 6.0 by adding sodium hydroxide and stored at 4°C. 0.1% BSA is not added until just before use because BSA encourages bacterial growth.

50 mM phosphate buffer, pH-7.4 - 50 mM Na_2HPO_4 (MW = 141.96) - 7.098 g/l + 50 mM $Na_2HPO_4.2H_2O$ (MW = 156.01) - 7.8 g/l. Na_2HPO_4 was brought to pH 7.4 by adding $Na_2HPO_4.2H_2O$. Na azide (5 ml) was added as a preservative and stored at 4°C.

1° Antibody - The 1° antibody was a gift from Dr. B. Williams of the Western General Hospital, Edinburgh. Our stock was a 1:100 dilution in 50 mM acetate buffer of the rabbit serum and it was stored frozen.

Donkey Anti-Rabbit Serum (DARS) - DARS was ordered from the Scottish Antibody Production Unit (SAPU) in 20 ml samples which were split to 2 ml aliquots and stored frozen. DARS was used as 1:10 dilution with Phosphate Buffer (+0.1% w/v BSA), and the diluted serum was never kept.

Normal Rabbit Serum (NRS) - NRS was also ordered from SAPU in 20 ml bottles but was split to 1 ml aliquots before storing frozen. NRS was used as 1:100 dilution with Phosphate Buffer and again the diluted serum was never kept.

Cyclic AMP Standards - A stock solution of cAMP (Sigma) in acetate buffer of 32 mM was used and stored at -20°C. Standards of 320, 32, 16, 8, 4, 2, 1, 0.5, 0.25, 0.125, and 0.0625 nM cAMP were made up in acetate buffer. Acetate buffer was used as the zero. Standards were stored frozen in their unacetylated state.

Acetylating Reagents - Triethylamine (TEA) and acetic anhydride (AA). The acetylating mixture consisted of 2:1 TEA to AA which was always made up in a glass tube and mixed well before use. The mixture was never stored but made up fresh before use and if TEA showed any signs of yellowing it was replaced

PEG 200 - PEG (Polyethyleneglycol) 200 (Aldrich)

Triton X-100 - Triton X-100 (Aldrich)

Background

In initial experiments, cAMP was measured using a cAMP assay that was already working in our laboratory which used a cAMP binding protein (BDH) to competitively bind cAMP and [^{3}H]cAMP (Armstrong *et al.*, 1985). However, the determination limit of this assay was not sufficiently low to detect any significant amounts of cAMP from the CHO cells (data not shown) and a new RIA was set up which was capable of measuring much lower concentrations of cAMP.

A primary antibody for acetylated cAMP was kindly made available to us from Dr. B. Williams, Department of Medicine, University of Edinburgh, Western General Hospital, Edinburgh. The only assays carried out with the 1° antibody used a charcoal solution to bind 'free' cAMP which was pelleted and counted after removing the supernatant. This assay method therefore counted the 'free' rather than the 'bound' cAMP and our preference was to set up an assay that would accurately measure the 'bound' fraction because of the very low levels of cyclic AMP being generated.

Before starting, dilution curves for the 1° antibody were run to determine the optimum concentration for the RIA. Aliquots (200 µl) of serially diluted 1° antibody from 1:1000 to 1:64000 were incubated overnight with ^{125}I-cAMP (50 µl, ~50000 cpm), DARS (50 µl, 1:3 dilution) and NRS (50 µl, 1:40 dilution). After centrifugation the supernatant was removed and the pellet counted which gave a curve for the dilution factor versus cpm (bound ^{125}I-cAMP) as shown in Figure 2.1.2a. From this a dilution factor of 1:20000 was chosen which produced ~50% binding as this would produce the most significant changes in cpm values for a small change in displacement. This was lowered to 1:40000 in later experiments to conserve the 1° antibody.

Once the dilution factor was determined, the assay was run and other factors optimised. One of these was the dilution of DARS and NRS to use. The initial protocol suggested mixing NRS and DARS at 1.5 ml NRS to 20 ml DARS, leaving the antibodies to bind overnight at 4°C before pelleting the conjugate, which was then resuspended in 50 ml

0.05 M phosphate buffer. This was attempted once and found to be inefficient and wasteful. Firstly, it was extremely difficult to resuspend the pellet evenly, because there was so much conjugate, and secondly, because the conjugate could not be refrozen, as it would breakdown the protein further, any unused conjugate could not be stored for more than a couple of days which meant discarding at least 50% after only one or two assays.

Another RIA assay protocol in use was one for $PGF_{2\alpha}$. In this protocol NRS and DARS dilutions were added in 50 µl aliquots to the assay tubes after they had been incubating with ligand and 1° antibody for 1 hr. This matched the protocol for incubation of ligand in my assay and suggested using dilutions of 1:3 for DARS and 1:40 for NRS. This worked well initially, but still meant a large consumption of serum especially the DARS, data not shown. Dilutions of 1:10 for DARS and 1:100 for NRS were therefore tested, and gave the best standard curves with a larger range, Figure 2.1.2b.

The final factor investigated in setting up this assay was the washing of tubes after overnight incubation before pelleting the conjugate, to limit non specific binding, and the method of discarding the supernatant. To determine the best washing conditions two standard curves were ran with being one washed with 1.5 ml 6% PEG 200 and the other with 1.5 ml 1% Triton X-100. The results are shown in Figure 2.1.2c and from these PEG 200 was chosen being more readily available and cheaper. To discard the supernatant there are a couple of methods: tubes can be 'tipped' and allowed to drip dry or the supernatant can be removed by aspiration. This assay used ^{125}I-cAMP as the radioactive source and aspiration was chosen as the method of removing supernatant.

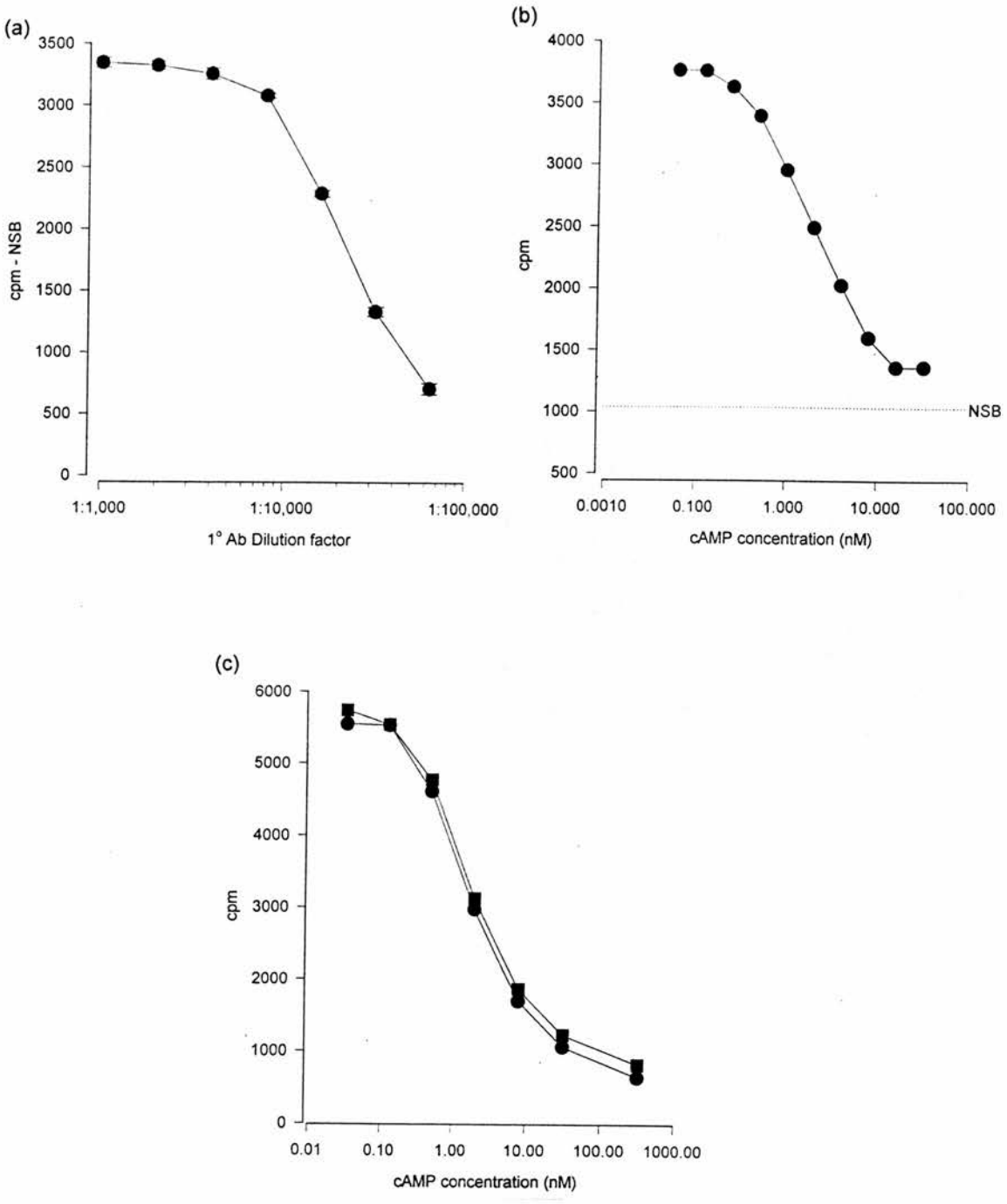

Figure 2.1.2.- (a) RIA dilution curve for 1°Ab (●) from which 1:20,000 was selected as the optimum dilution factor, n=4. (b) An example RIA standard curve (●), n=1. (c) The effects of washing with PEG 200 (●) or Triton X-100 (■) and PEG 200 was chosen as the wash solution, n=1.

RIA protocol

1 - 500 µl aliquots of samples and standards were acetylated with 20 µl of the 2:1 TEA/AA mixture, whirlimixed immediately and left for 2-3 min to acetylate.

2 - The number of assay tubes required was calculated, e.g. have 82 unknown samples. Assayed in duplicate = 164 tubes.
Need 164 + 28 (standard curve + total counts) = 190 tubes.

3 - The volume of ^{125}I-cAMP/1° antibody mixture was determined, e.g. 190 tubes in the assay (round up to 200) 200 x 200 µl = 40 ml.
Measured out 40 ml of acetate buffer and added 0.1% w/v BSA.

4 - ^{125}I-cAMP was counted and a volume calculated that gave ~5000 cpm/tube, e.g. 10 µl ^{125}I-cAMP = 2000000 cpm
With a 200 tube assay require 200 x 5000 = 1000000 cpm, so added 5µl of ^{125}I-cAMP ligand to 40 ml acetate buffer

5 - Volume of 1° antibody required was determined, e.g. used 1:20000 end dilution. Stock was 1:100, so added 200 µl of 1:100 stock to 40 ml buffer = 1:20000 dilution

6 - The ^{125}I-cAMP/1° antibody mixture was sealed and vortexed. This must be made up quickly, mixed well and used immediately otherwise the ^{125}I-cAMP has time to non-competitively bind to the 1° antibody.

7 - The reagents were added to the tubes as shown in Table 2.1.1.

8 - All tubes were incubated for 1 hr at 4°C.

9 - 50 µl of DARS (1:10 dilution in 50 mM phosphate buffer) and 50 µl of NRS (1:100 dilution in 50 mM phosphate buffer) were added.
The tubes were vortexed, and incubated overnight at 4°C.

10 - 1.5 ml 6% PEG was added to all tubes, except T/T. The tubes were then vortexed and spun for 30 min at 1900g, 4°C.

11 - The supernatant was removed.

12 - All tubes were counted, including T/T, for 1 min on a Beckman γ-counter. The counter was pre-programmed to plot a standard curve from the standards and to calculate a best-fit hyperbola from the points using a 'Logit' fit of log x versus y. From this curve the counter also calculated the unknowns in fmol of cAMP present. As experiments were carried out in duplicate and assayed for cAMP in duplicate, the program also calculated the mean result.

Tube No.	Volume added		Definition
	Standard/ Unknown	^{125}I-cAMP/ 1° Ab mix	
T/T*	-	200 µl	Total counts
1/2/3	50 µl	200 µl	No cAMP (Total bound)
4/5/6	"	"	NSB
7/8	"	"	0.0625 nM cAMP
9/10	"	"	0.125 nM cAMP
11/12	"	"	0.25 nM cAMP
13/14	"	"	0.5 nM cAMP
15/16	"	"	1.0 nM cAMP
17/18	"	"	2.0 nM cAMP
19/20	"	"	4.0 nM cAMP
21/22	"	"	8.0 nM cAMP
23/24	"	"	16.0 nM cAMP
25/26	50 µl	200 µl	32.0 nM cAMP
27/28	50 µl	200 µl	Unknown
29/30	50 µl	200 µl	Unknown
.....	50 µl	200 µl	Unknown

Table 2.1.1. - cAMP RIA setup

* - tubes containing total radioactivity, these are not centrifuged and allow a calculation of the degree of binding of 1° antibody

All values are expressed as increases from basal. To obtain this, the blank cell value was subtracted from each sample in each experiment before the results were pooled to give average ± SEM.

EEC values were calculated for each experiment by dividing the drug concentration that produces a 50 or 30% maximal response by the PGE_2 concentration that produces an equivalent response.

pA_2 values are calculated as the average -log K_B values for each experiment. These are "apparent" pA_2 values, as they assume a Schild plot with a gradient of 1. For AH23848B, it was not possible to calculate the Schild gradient as the only effective concentration was the maximum available concentration of 30 μM.

2.1.4.- Results

Time course

Initially a time course was run to determine the optimum incubation time for sampling cAMP generation. PGE_2 1 μM produced a maximum cAMP production in the cellular fraction after 2 min of 1010 ± 260 fmol cAMP/10^5 CHO cells which decreased after 30 min to 770 ± 210 fmol cAMP/10^5 CHO cells, n=4, Figure 2.1.3. However, the maximum production observed in the medium after 5 min incubation was only 210 ± 170 fmol cAMP/10^5 CHO cells, n=4, Figure 2.1.3. Five min was chosen as the optimum incubation time with all samples taken from the cellular fraction.

Antagonist and partial agonist studies

AH23848B 30 μM reduced the concentration-dependent production of cAMP by PGE_2, n=6, Figure 2.1.4a. This inhibition was not significant when analysed by ANOVA but point analysis using a paired two-tailed Student *t*-test show significant differences for 100 nM and 10 μM PGE_2 ($p<0.05$). These data suggest the involvement of the EP_4 receptor subtype in mediating the production of cAMP by PGE_2.

An "apparent" pA_2 value for AH23848B of 6.2 was calculated from K_B values using the formula:

$$CR-1 = [B]/K_B ,$$

where [B] is the concentration of the antagonist and CR is the Concentration Ratio.

Butaprost was examined for antagonist activity in this preparation. Butaprost was used at 10 μM, the maximal concentration used as an agonist, and did not significantly reduce the concentration-dependent increases in cAMP observed with PGE_2, n=4, Figure 2.1.4b. Statistical analysis of this data with ANOVA and paired Student *t*-test for each point showed no significant effect, $p>0.05$. Interestingly, though,

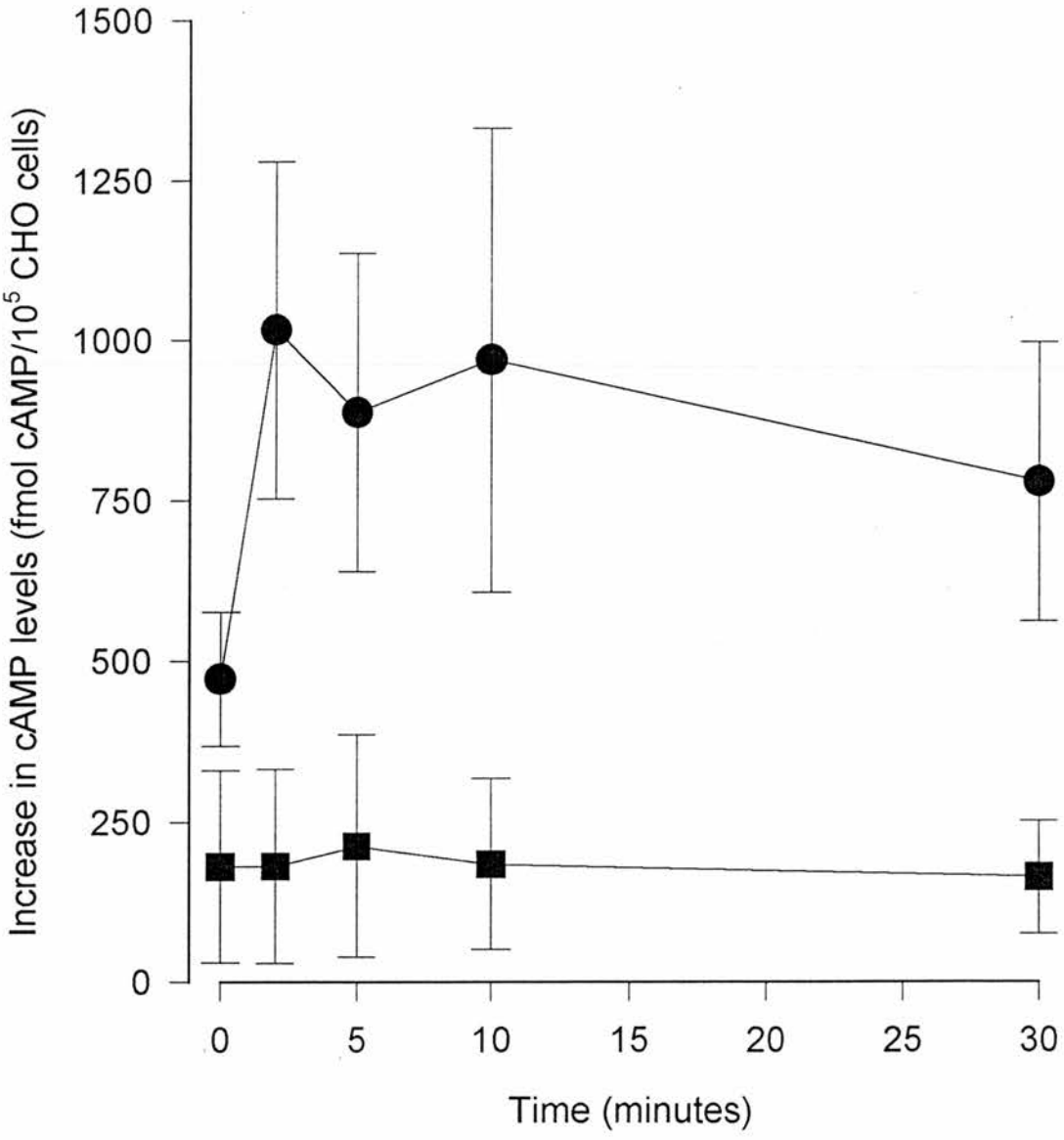

Figure 2.1.3.- Time course of PGE_2 (1 μM) stimulated cAMP production in CHO cells (●) and medium (■), n=4. 2 min incubation produced a maximum increases of cAMP in the cellular fraction of 1010 ± 260 fmol cAMP/10^5 CHO cells. 5 min was selected as the incubation time for all subsequent experiments.

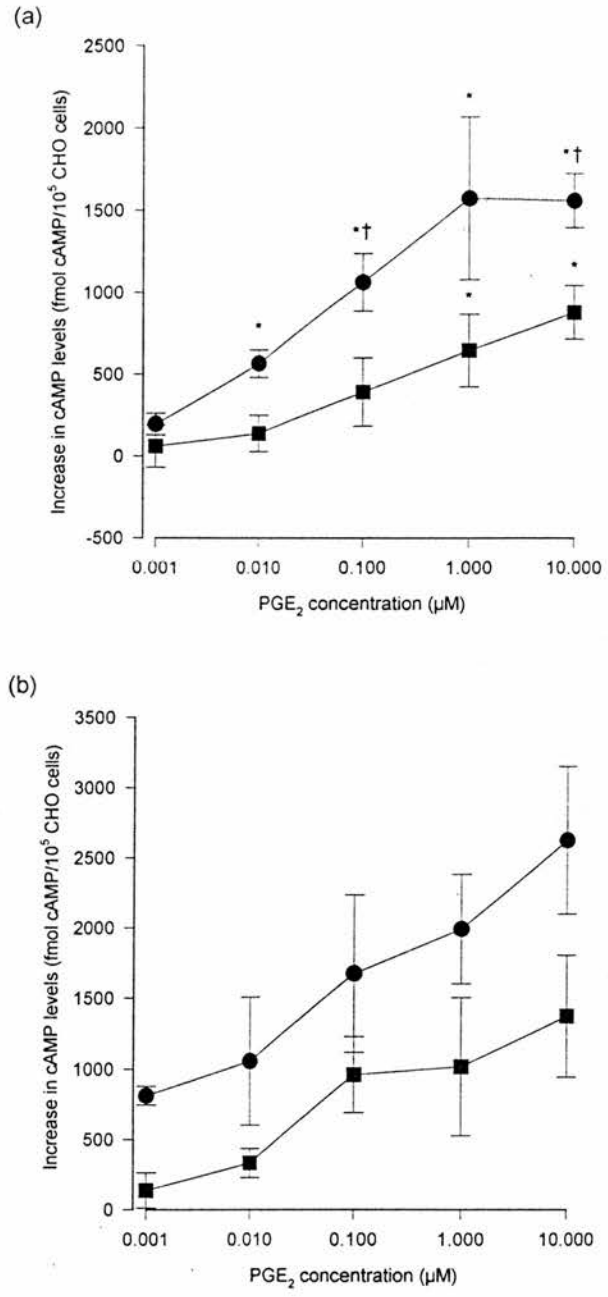

Figure 2.1.4.- PGE_2-induced increases in cAMP levels in CHO cells. (a) Effect of PGE_2 in the absence (●) and presence (■) of AH23848B (30 μM), n=6 ($p>0.05$ with ANOVA), and (b) Effect of PGE_2 in the absence (●) and presence (■) of butaprost (10 μM), n=4 ($p>0.05$ with ANOVA). * - $p<0.05$ compared with basal production, † - $p<0.05$ between control and AH23848B, using paired two-tailed Student *t*-test.

butaprost did produced a trend towards the right in the concentration-effect curve for PGE_2.

Effect of agonists at increasing intracellular cAMP

PGE_2 increased cAMP generation in CHO cells in a concentration-dependent manner with an EC_{30} of 21 ± 12 nM and an EC_{50} of 94 ± 28 nM, maximal production of 1980 ± 270 fmol cAMP/10^5 CHO cells at a concentration of PGE_2 10 μM, n=7, Figure 2.1.5a. PGE_2 produced significant increases in cAMP from basal at 10 nM and greater concentrations ($p<0.05$, two-tailed paired Student *t*-test).

16,16-dimethyl PGE_2 and 11-deoxy PGE_1 also increased cAMP in CHO cells in a concentration-dependent manner, Figure 2.1.5a. 16,16-dimethyl PGE_2 produced significant increases in cAMP at 1-10 μM concentrations with an EC_{30} of 560 ± 260 nM, n=4, and an EC_{50} of 2.5 ± 1.3 μM, n=3. 11-deoxy PGE_1 only significantly increased cAMP at 10 μM with an EC_{30} of 430 ± 230 nM and an EC_{50} of 5.2 ± 2.4 μM, n=4. 11-deoxy PGE_1 and 16,16-dimethyl PGE_2 gave EEC values of 134 and 163 with respect to PGE_2. Although these EEC values are determined relative to PGE_2 and indicate that these compounds have a lower potency, when taken relative to their own maximal responses 16,16 dimethyl PGE_2 produced an EC_{50} of 110 ± 40 nM and 11-deoxy PGE_1 190 ± 60 nM which are similar to that of PGE_2 (EC_{50} = 94 ± 28 nM) and suggest these compounds could be partial agonists at the EP_4 receptor.

Neither AH13205 nor butaprost significantly increased cellular cAMP above basal in the CHO cell line, Figure 2.1.5b. AH13205 and butaprost produced maximal increases of 150 ± 180 and 100 ± 110 fmol cAMP/10^5 CHO cells respectively. This suggests that EP_2 receptors are not involved in mediating cellular increases in cAMP in the CHO cell line.

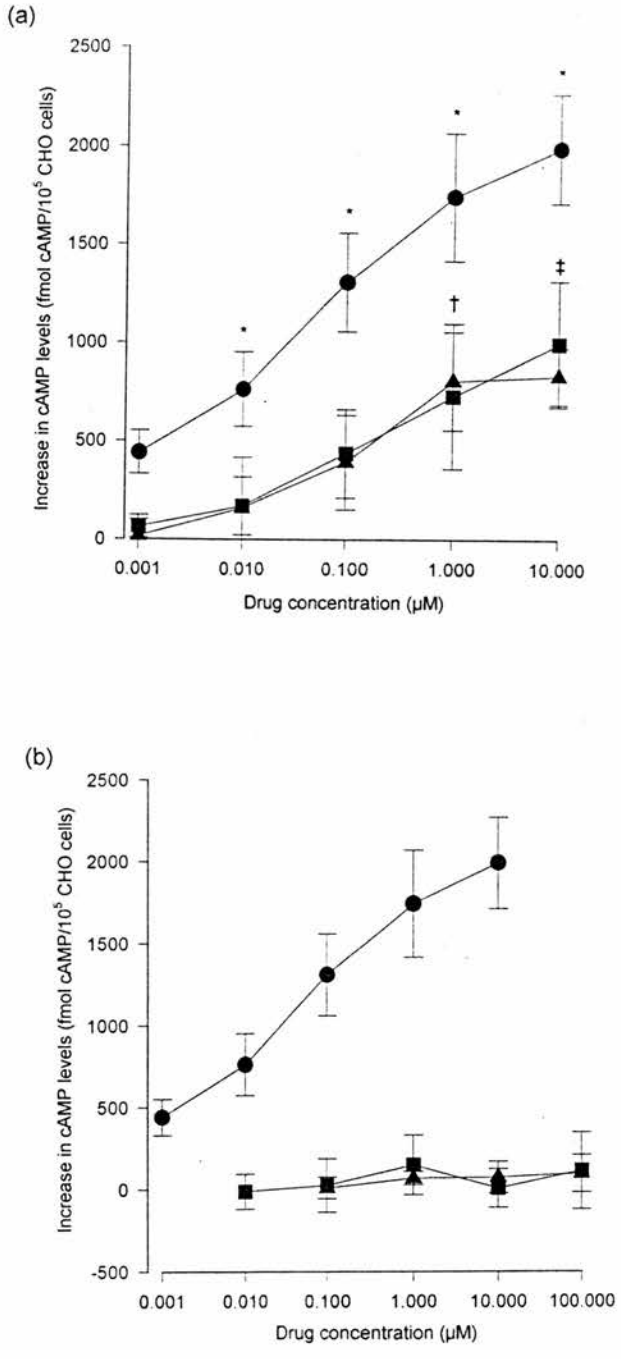

Figure 2.1.5.- (a) Effect of PGE_2 (●), n=10, 11-deoxy PGE_1 (■), n=4, and 16,16-dimethyl PGE_2 (▲), n=4, and, (b) effect of PGE_2 (●), n=10, AH13205 (■), n=4, and butaprost (▲), n=4, at increasing cellular cAMP in the CHO cell line.

* - $p<0.05$ for PGE_2 , † - $p<0.05$ for 16,16 dimethyl PGE_2 and ‡ - $p<0.05$ for 16,16 dimethyl PGE_2 and 11-deoxy PGE_1 compared with basal production using paired two-tailed Student *t*-test.

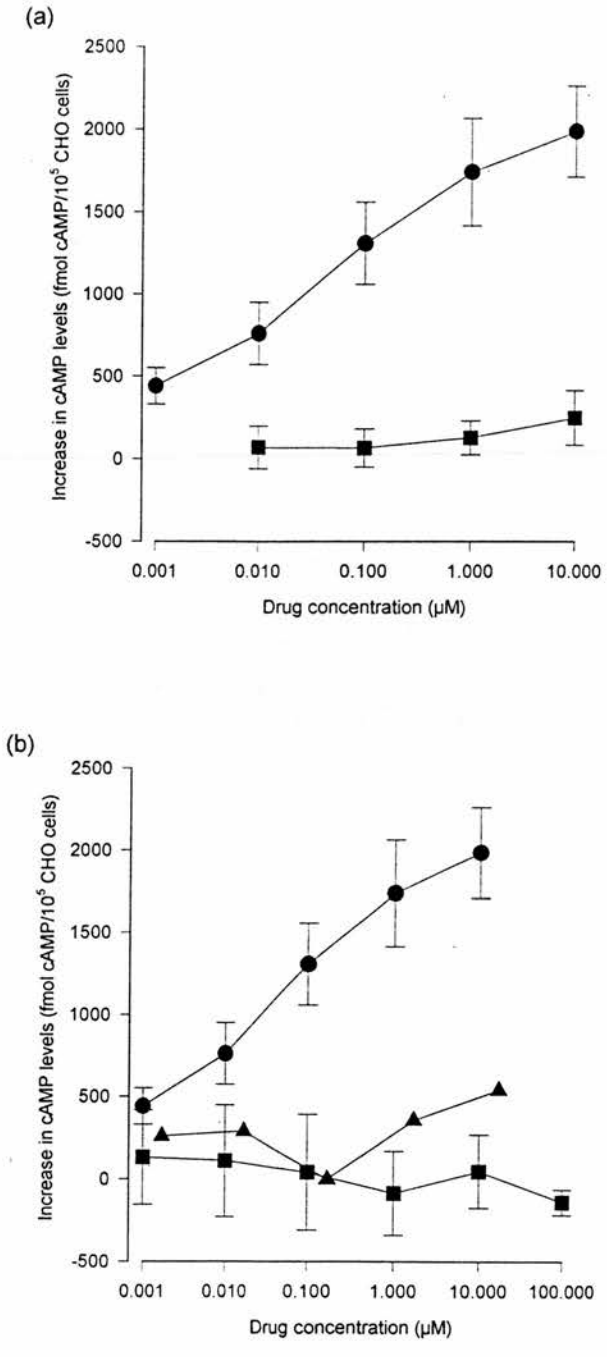

Figure 2.1.6.- (a) Effect of PGE_2 (●), n=10, and misoprostol (■), n=4, and, (b) effect of PGE_2 (●), n=10, sulprostone (■), n=3, and GR63799X (▲), n=1, at increasing cellular cAMP in the CHO cell line.

Misoprostol, sulprostone and GR63779X had no significant effect on cellular cAMP generation, Figure 2.1.6a & b. Sulprostone tended to produce a very small reduction in basal cAMP levels which may have been due to its activation of EP_3 receptors negatively coupled to AC. However that was not significant. Misoprostol has agonist activity at EP_2 receptors and its inactivity here further suggests that the PGE_2-induced increases in cAMP are not mediated via EP_2 receptors.

The naturally-occurring prostaglandins, PGD_2 and $PGF_{2\alpha}$, produced no significant increases in cAMP, Figure 2.1.7a & b.

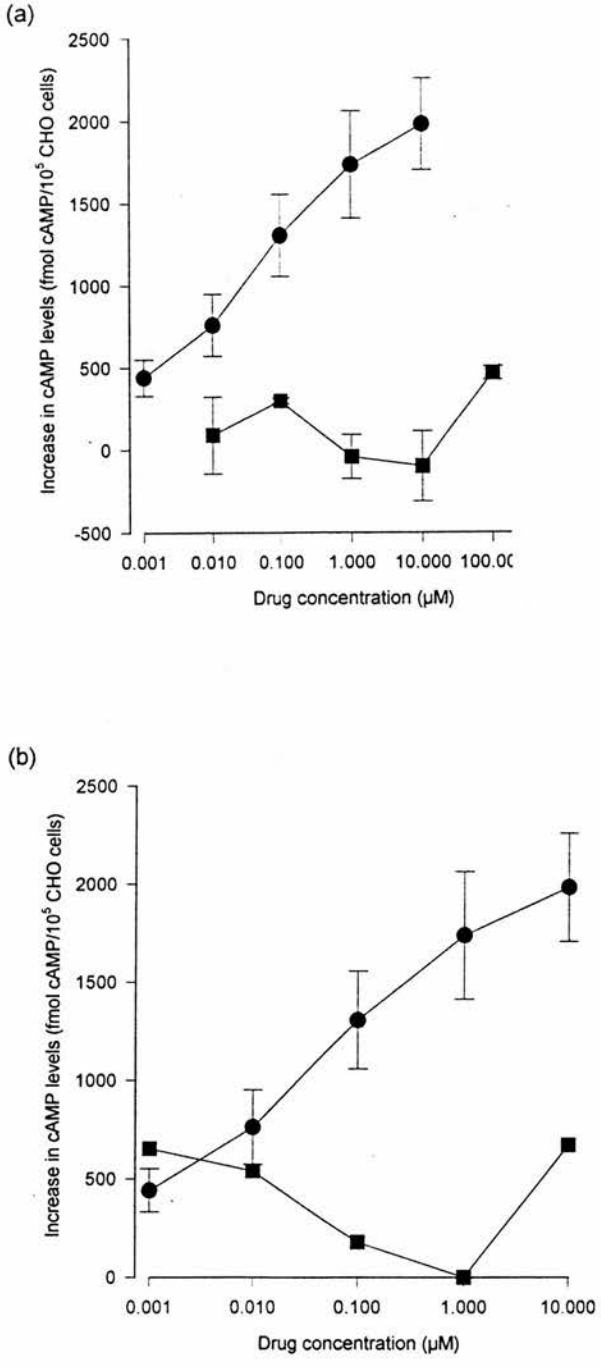

Figure 2.1.7.- (a) Effect of PGE_2 (●), n=10, and $PGF_{2\alpha}$ (■), n=3, and, (b) effect of PGE_2 (●), n=10, and PGD_2 (■), n=1, at increasing cellular cAMP in the CHO cell line.

2.1.5.- Discussion

AH23848B was used as an EP_4 antagonist to determine the effects of EP_4 receptor antagonism on cAMP production in CHO cells stimulated with PGE_2. AH23848B produced a slight rightward shift in the concentration-response curve for PGE_2 with an apparent pA_2 of 6.2, Figure 2.1.4a. This is higher than the pA_2 of 5.4 observed in the PSV (Louttit *et al.*, 1992a) and 5.3 in transfection studies using the cloned EP_4 receptor (Nishigaki *et al.*, 1995). Nevertheless, these data support the involvement of the EP_4 receptor in the generation of cAMP by PGE_2 in the CHO cell.

Butaprost was examined for antagonist activity, and tended to shift the PGE_2 concentration-response curve to the right. However, this effect was not statistically significant and provides only an interesting observation.

11-deoxy PGE_1 and 16,16 dimethyl PGE_2 concentration-dependently increased cAMP in the CHO cell, Figure 2.1.5a, whereas AH13205 and butaprost produced no significant increases in cAMP levels, Figure 2.1.5b. Interestingly, when the EC_{50} values for 16,16 dimethyl PGE_2 and 11-deoxy PGE_1 were calculated relative to their own maxima, they produced similar values to PGE_2 of about 100 nM, which suggests that these two compounds could be partial agonists at the EP_4 receptor expressed in CHO cells. The assumption made here, however, is that all three compounds have reached their respective maximum responses but, when looked at in Figure 2.1.5a, only the concentration-response curve for 16,16-dimethyl PGE_2 has reached a plateau, and that higher concentrations of both PGE_2 and 11-deoxy PGE_1 might produce even greater increases in cellular cAMP and, thus, increased EC values.

Together with the effect observed for AH23848B in this cell line, these results suggest that the EP receptor positively coupled to AC in the CHO cell is not the EP_2 receptor subtype but rather the EP_4, and that 11-deoxy PGE_1 and 16,16 dimethyl PGE_2 have agonist activity at the EP_4 receptor.

Further to these observations, Honda and co-workers report that at the EP_4 receptor, originally classified as EP_2, butaprost does not displace bound [^{3}H]PGE_2 (Honda *et al.*, 1993). This is followed up by observations in the same transfected cell line that butaprost does not stimulate cAMP generation (Nishigaki *et al.*, 1995). They also observed that AH23848B displaces [^{3}H]PGE_2 binding and antagonised PGE_2-stimulated cAMP formation. Interestingly, this mouse EP_4 receptor has been transfected in the CHO cell line, and although it is shown here that these cells endogenously express the EP_4 receptor the levels of receptor expression is probably far greater in the transfected cells. Reported levels of cAMP generation are greater than those observed in our studies, for example PGE_2 10 μM induces an increase of about 20 pmol/10^5 cells in the transfected CHO cell compared to only 2 pmol/10^5 cells in the native CHO cells.

Misoprostol produced no significant increases in cAMP over basal levels, Figure 2.1.6a. It is interesting to note, though, that Honda and colleagues observe almost complete displacement of [^{3}H]PGE_2 from their cloned EP_4 receptor with misoprostol (Honda *et al.*, 1993), and use this information to conclude that their expressed receptor is the EP_2 receptor subtype, irrespective of a complete inactivity observed with butaprost. This information suggests that misoprostol has a high binding affinity for the EP_4 receptor, about 50% displacement of [^{3}H]PGE_2 was observed with PGE_2 10 nM and misoprostol 100 nM (personal estimation from Figure 2, Honda *et al.*, 1993), but has limited, if any, efficacy at this CHO cell EP_4 receptor.

Sulprostone and GR63799X had no effect on cAMP levels in the CHO cell, Figure 2.1.6b. This does not rule out any expression of EP_1 or EP_3 receptors in the CHO cell line, but rather shows that these compounds have no agonist activity at the EP_4 receptor endogenously expressed by these cells. $PGF_{2\alpha}$ and PGD_2 both increased cAMP levels in the CHO cell at their highest concentrations of 1 mM and 10 μM respectively, Figure 2.1.7a & b. These are probably due to cross reactivity with the EP_4 receptors expressed in these cells as Honda and co-workers, 1993, observed almost complete displacement of [^{3}H]PGE_2 from the COS-1 cell membranes by $PGF_{2\alpha}$ 10 μM and PGD_2 10 μM (Honda *et al.*, 1993).

It seem likely that the CHO cell line endogenously expresses an EP receptor positively coupled to AC and cAMP generation that resembles the EP_4 receptor proposed by Louttit and co-workers in 1992 (Louttit *et al.*, 1992a). This work has been further supported by studies involving cloned EP receptors. Regan and colleagues have cloned the human EP_2 receptor subtype and shown that butaprost and AH13205, selective EP_2 agonists, produced concentration-dependent increases in cAMP as well as displacing bound $[^3H]PGE_2$. Further to this, cloning and expression of the mouse EP_4 receptor in COS-1 cells by Namba and co-workers (Namba *et al.*, 1993) and the further stable expression in CHO cells (Nishigaki *et al.*, 1995), show inactivity of butaprost, and another selective EP_2 agonist 19(R)OH-PGE_2 (Woodward *et al.*, 1993), to displace bound $[^3H]PGE_2$ and increase intracellular cAMP levels. Furthermore, Nishigaki and colleagues also observe antagonism of PGE_2-stimulated cAMP accumulation by AH23848B at the cloned EP_4 receptor.

In conclusion, the CHO cell endogenously expresses the EP_4 receptor which is positively coupled to AC activation and generation of intracellular cAMP. This is the first published observation to link the native EP_4 receptor with AC activation, and these results demonstrate that the CHO cell line offers a good *in vitro* system in which to study the EP_4 receptor. However, one of the limitations of this system is the small increase in cAMP generation, possibly due to a small receptor number. Also, unfortunately, we did not correlate changes in cAMP with a physiological response in CHO cells since we did not have access to the required technology to measure responses such as cell acidification or Ca^{2+} flux.

CHAPTER 2.2

Further characterisation of the EP_4 receptors present on the pig saphenous vein, and attempted classification of the rabbit jugular vein, relaxant EP-receptor(s)

2.2.1.- Introduction

The original classification of EP-receptor was based largely on data obtained using smooth muscle preparations, and demonstrated four subtypes. Typically, EP_1 receptors mediate contraction (e.g., guinea-pig gastric fundus) and EP_2 receptors mediate relaxation of smooth muscle (e.g., rabbit ear artery, cat trachea), via activation of PLC, and AC, respectively. EP_3 receptor activation is more varied and includes inhibition of neuronally-mediated contractions (e.g., guinea-pig vas deferens), potentiation of platelet aggregation, inhibition of gastric acid secretion, and contraction of some smooth muscle preparation (e.g., chick ileum), linked either to activation of phospholipase C or inhibition of AC. The most recently described subtype, EP_4 (Louttit *et al.*, 1992a), is a relaxant EP receptor coupled to AC activation (Honda *et al.*, 1993; Nishigaki *et al.*, 1995)

The RJV has been used extensively as a model preparation in which to study prostanoid receptors, and has been shown to contain DP (Giles *et al.*, 1989), IP (Giles *et al.*, 1990) and TP receptors (Lumley *et al.*, 1989). The RJV also relaxes in response to PGE_2 and is currently classified as an 'atypical EP_2' preparation, based on the low potency of the EP_2 agonist butaprost (Lawrence & Jones, 1992).

Our interest in this study was to classify the relaxant EP receptor present in the RJV from the perspective of anti-glaucoma therapy. Drugs currently available for topical use to reduce intraocular pressure (IOP) in glaucoma patients include adrenoceptor agonists, (e.g. adrenaline), cholinoceptor agonists (e.g. pilocarpine) and β-adrenoceptor antagonists (e.g. timolol). The mechanism of action by which these currently used therapies for glaucoma treatment reduce IOP are not known. There is evidence which suggests that the antiglaucoma effects of adrenaline may be mediated via stimulation of PG synthesis. Indomethacin, an inhibitor of PG synthesis, was shown to inhibit the hypotensive response to chronically administered adrenaline in both the rabbit and human eye (Bhattacherjee & Hammond, 1977; Camras *et al.*, 1985), and adrenaline has been shown to increase AA release and PG synthesis by the iris-ciliary body both *in vitro* (Yousufzai & Abdel-Latif, 1983, 1984) and *in vivo* (Yousufzai & Abdel-Latif, 1987). It has now been shown that PGs

themselves can produce a significant and prolonged reduction in IOP, and can increase aqueous outflow (Camras *et al.*, 1977; Stern & Bito, 1982; Lee *et al.*, 1984). PGD_2 has been shown to lower IOP in rabbits (Woodward *et al.*, 1990) as has PGA_2-1-isopropylester in cats (Bito *et al.*, 1990). $PGF_{2\alpha}$, or its isopropylester derivative, $PGF_{2\alpha}$-isopropylester, though, are more potent ocular hypotensive agents in rabbits (Camras *et al.*, 1977; Lee *et al.*, 1984), cats (Stern & Bito, 1982), dogs (Groeneboer *et al.*, 1989), monkeys (Camras & Bito, 1981; Stern & Bito, 1982; Lee *et al.*, 1984; Crawford *et al.*, 1987; Crawford & Kaufman, 1991) and humans (Kerstetter *et al.*, 1988; Lee *et al.*, 1988), and can reduce IOP when applied topically to patients with glaucoma (Camras *et al.*, 1989). However, the mechanism underlying these PG effects remains to be elucidated.

$PGF_{2\alpha}$ and PGE_2 are the two major PGs produced by ocular tissue (Abdel-Latif, 1991), but information about the functional aspects of PGs and the relevant receptors in the eye is sparse. It has been demonstrated that iris sphincter muscles of cats and dogs contain predominately FP receptors, (Kennedy *et al.*, 1982), whereas those of bovine iris sphincter muscle are predominately EP receptors (Dong *et al.*, 1986). Autoradiographic imaging of receptors in human eyes with $[^3H]PGE_2$ and $[^3H]PGF_{2\alpha}$ have shown co-localisation at a high level in the areas of the ciliary muscles and iris sphincter muscles (Matsuo & Cyander, 1992). Matsuo & Cyander, 1992, suggest that $PGF_{2\alpha}$ and PGE_2 could modulate uveoscleral outflow, thus lowering IOP, by binding to their receptors located on the ciliary muscles and inducing their relaxation. However, Woodward *et al.*, 1989, showed, with a range of compounds, that the rank order of potency for decreasing IOP was $PGF_{2\alpha}$ > $PGF_{1\alpha}$ > 16-phenoxytetranor $PGF_{2\alpha}$ > 17-phenyl trinor $PGF_{2\alpha}$ = fluprostenol (inactive) whereas that for FP receptor selectivity was the reverse implying that the conventional FP receptor is not involved in lowering IOP (Woodward *et al.*, 1989). TP receptor activation has also been shown to have no effect on IOP in cats (Bito, 1984).

A study in rabbits implicates the involvement of the EP_3 receptor subtype in lowering IOP (Waterbury *et al.*, 1990). Sulprostone and two analogues RS-61565 and RS-20216, lowered IOP up to a maximum of

12 hr in rabbits after topical administration. In contrast U46619 had no effect, whereas PGE_1, and PGE_2, both induced a hypertensive response before eliciting a decrease in IOP. This has been supported by a study using more selective prostaglandin analogues and comparing radioligand binding studies with functional assays (Woodward *et al.*, 1994). The group concluded that EP_3 and FP receptors co-existed as discrete entities and activation of either, or both, lowered IOP.

An interesting point to note is that short term desensitisation of $PGF_{2\alpha}$ receptors, by a 30 min pre-incubation with $PGF_{2\alpha}$, in the bovine iris sphincter, a species which produces very little ocular PGs, increased cAMP formation and reduced IP_3 production and muscle contraction induced with $PGF_{2\alpha}$ (Yousufzai *et al.*, 1989). In bovine iris sphincter, Tachado and co-workers demonstrated that preincubation with $PGF_{2\alpha}$ results in AC activation upon further challenge with $PGF_{2\alpha}$ (Tachado *et al.*, 1993). They suggest that $PGF_{2\alpha}$ induced desensitisation might uncouple the FP receptor from the G_q and G_i proteins and enhance $PGF_{2\alpha}$ mediated stimulation of AC activity through G_s. Recent work shows that PGD_2, PGE_2, and $PGF_{2\alpha}$ to a lesser extent, added exogenously to bovine ciliary muscle induced increases in cAMP formation (Yousufzai *et al.*, 1994). In species which produce large amounts of ocular PG, it is possible that normal G_q and G_i coupled receptors are desensitised.

Irrespective of the receptor system which lowers IOP, a hindrance to drug development in this field has been that $PGF_{2\alpha}$, and other compounds which lower IOP, have the undesirable side-effect of causing hyperaemia, redness around the eye (Protzman & Woodward, 1990). Attempts have been made to synthesise analogues which induce minimal hyperaemia while still retaining the ability to effectively lower IOP. Studies by D.W. Woodward (personal communication), found that compounds which reduced IOP but induced hyperaemia were capable of relaxing the RJV, whereas, compounds which reduced IOP but showed little hyperaemia, did not relax the RJV. Thus it was decided to try to identify the receptor which mediated $PGF_{2\alpha}$-induced relaxation of the RJV and, in particular, to establish if $PGF_{2\alpha}$ induces relaxation of RJV by interaction with a

discrete FP receptor or by interacting with the atypical EP receptor previously described. The nature of the 'atypical EP_2' receptor on the RJV was also investigated to see if it could be characterised as an EP_4 receptor. The PSV was used as a known EP_4 receptor containing preparation to confirm and extend previous findings.

The aim of the present study was to examine in detail and compare the pharmacology of prostanoid-induced relaxation of the isolated PSV and RJV by determining the activity of prostaglandin agonists with selectivity for specific prostanoid receptors. Compounds examined along with PGE_2 in these preparations include 16,16-dimethyl PGE_2 (Lawrence & Jones, 1992), 11-deoxy PGE_1 (Banjeree *et al.*, 1985), AH13205 (Nials *et al.*, 1991), butaprost (Gardiner, 1986) and nocloprost (Tauber *et al.*, 1988). Possible antagonism by AH23848B, AH6809 (Keery & Lumley, 1988), and BW A868C (Giles *et al.*, 1989) has also been investigated along with the effects of nitric oxide with a stable nitric oxide synthase inhibitor L-N^G-monomethyl arginine (L-NMMA) (Palmer *et al.*, 1988).

2.2.2.- Methods

Materials

Krebs Solution - Krebs solution was prepared either on the morning of the experiment or the day before, in which case no $CaCl_2$ was added until just before the experiment, after storage overnight at 4°C.
The composition was in mM;

$MgSO_4$	1.0
NaH_2PO_4	1.1
$CaCl_2$	2.5
KCl	5.4
glucose	10
$NaHCO_3$	25
NaCl	118

N.B. It was important to add the $CaCl_2$ after dissolving the chemicals completely and to add it slowly allow it to mix well. If the Krebs solution turned cloudy after adding the $CaCl_2$ it was discarded and the flask washed thoroughly before making up new Krebs solution.
Sagatal - Pentobarbitone Sodium B.P. 60mg/ml, made up as a 1:1 solution with 0.9% saline.
Heparin - Heparin Sodium (Mucous) 5000 units/ml, made up as 1:100 solution with 0.9% saline.
Drugs - All drugs were prepared fresh each day in 0.9% NaCl from ethanol or dry powder stocks stored at -20°C. AH23848B was sonicated for 30 min before use.

Pig Saphenous vein

Saphenous veins were taken from Chinese Meishen pigs which had been killed by intracardiac injection of pentobarbitone. An incision was made in the skin proximal to the ankle joint and the saphenous vein excised into Krebs solution. The veins were stripped of all fat and connective tissue before use. The vessel was cut into four equal pieces of 3-5 mm in width, suspended in an overflow organ bath between two silver alloy hooks at 2-3 g tension and washed with Krebs solution containing indomethacin (1 μM), bubbled with O_2/CO_2 (95/5 v/v) and maintained at 37°C. Changes in tension were measured using Grass

FT03 isometric transducers connected to a Maclab® data acquisition system using Maclab® v3.3 software. All drugs were added in 100 µl aliquots to the bath and all concentrations quoted are bath concentrations.

The experimental procedures for PSV and RJV were essentially the same except for the fact that phenylephrine was used as a contractile stimulus for the PSV, as for Louttit *et al.*, 1992a,b, whereas histamine was used for the RJV. Initially, histamine was used as a contractile agent for both preparations since this would give a more direct comparison between the two, unfortunately histamine did not induce consistent contractions of the PSV, sometimes these were huge which were impossible to reverse when washing out, or they were phasic, in a fashion which was difficult to stabilise. The second protocol change was the addition of adenosine deaminase (1U/ml) to PSV organ baths before phenylephrine to limit fade of the tissue. It has been observed before that the PSV spontaneously releases adenosine upon stimulation, and that adenosine is a vasodilator relaxing the vessel and potentiating the effects of any relaxant compounds. It was important to remove this effect and 1U/ml of adenosine deaminase was selected as a high enough concentration to be effective without disrupting the viability of the tissue.

Phenylephrine was added as the stimulating agent at an initial concentration of 10 µM to determine the maximal contraction of the tissue. A concentration was then chosen which gave 60-80% of this maximal response which was usually 0.5-1 µM (range 0.1-2 µM). When each phenylephrine induced contraction plateaued, cumulative concentrations of a PG analogue were added to the organ bath, with each response being allowed to plateau before adding the next concentration. All compounds were tested in the presence of GR32191X (10 µM) to block any contractile action at TP receptors (Lumley *et al.*, 1989).

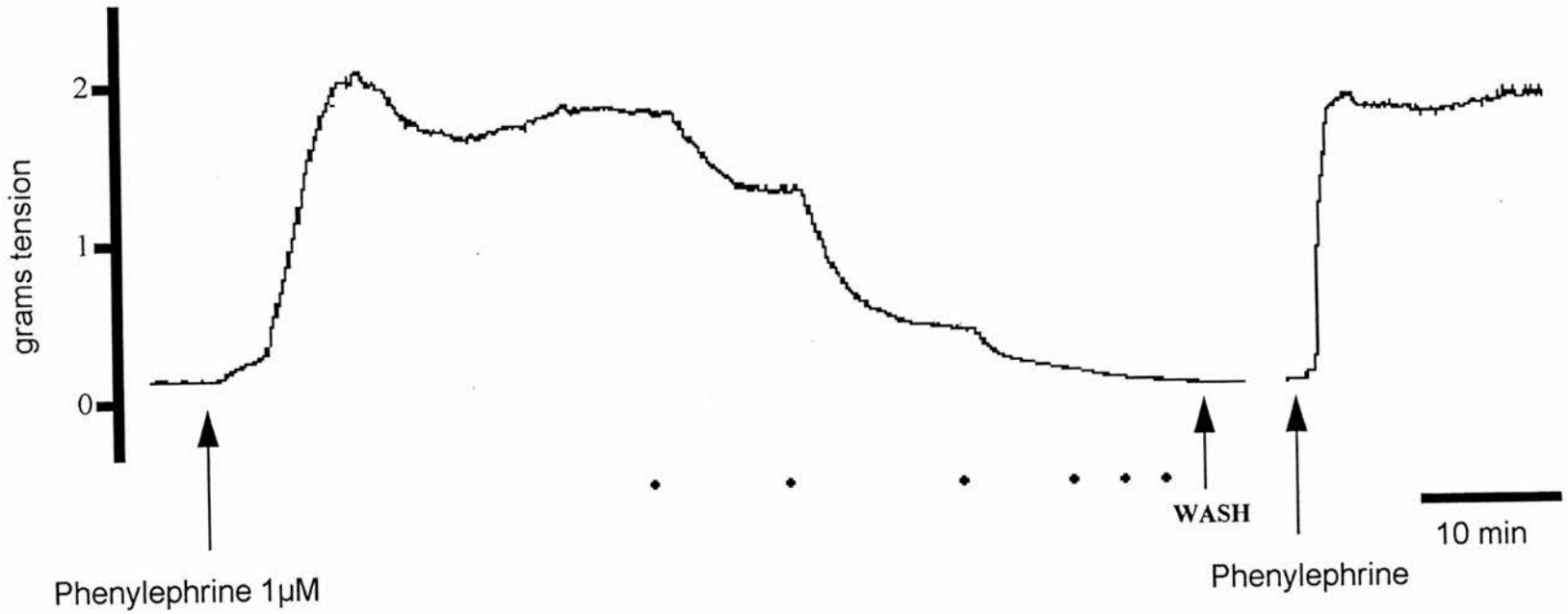

Figure 2.2.1.- Example concentration-response curve in the PSV for PGE_2 (0.1 nM- 30 nM). All concentration-response curves were carried out at 37°C in Krebs solution with GR32191X 10 µM, indomethacin 1 µM and adenosine deaminase 1 U/ml.
•- addition of PGE_2 (0.1- 30nM)

Rabbit Jugular Vein

Male New Zealand white rabbits (2-3 kg) were anaesthetised with 30 mg/kg Sagatal (pentobarbitone) injected into the marginal ear vein and Sagatal administered as required to ensure that the animal remained fully anaesthetised. The external jugular veins were then exposed on either side of the trachea, after shaving and opening the neck, and tied at the proximal end. Once the vessels had filled with blood they were tied at the distal end before excising into a beaker of Krebs solution. The inflated sections (2-3 cm in length) were first cleared of fat and connective tissue in a petri dish filled with Krebs solution before cutting into 3-5 mm sections for the experiment. These were suspended as for the PSV but at a lower tension of 0.75-1.25 g.

After equilibrium of the tissues for 1 hr, the maximum contraction to histamine (10 μM) was measured, and a concentration was selected, usually 1 μM (range 0.1-5 μM), that produced 60-80% of the maximum concentration. Cumulative concentration-response curves for a range of prostanoid receptor agonists were then carried out, as described above, in the presence of GR32191X (10 μM).

Fade was an important consideration in both preparations, and in each experiment, all preparations were left in the presence of a submaximally effective concentration to observe the effects of fade. Very often one or two of the tissues would show extreme fade but still be included in the experiment in case they stabilised. If the tissues did not stabilise, the data obtained from these preparations would not be used in the analysis. Adenosine deaminase extensively limited fade in the PSV, but fade still remains a problem with this experimental set-up. If a tissue exhibits slight fade and is not noted while running the experiment then the effects of any relaxant PG analogue will be greatly exaggerated.

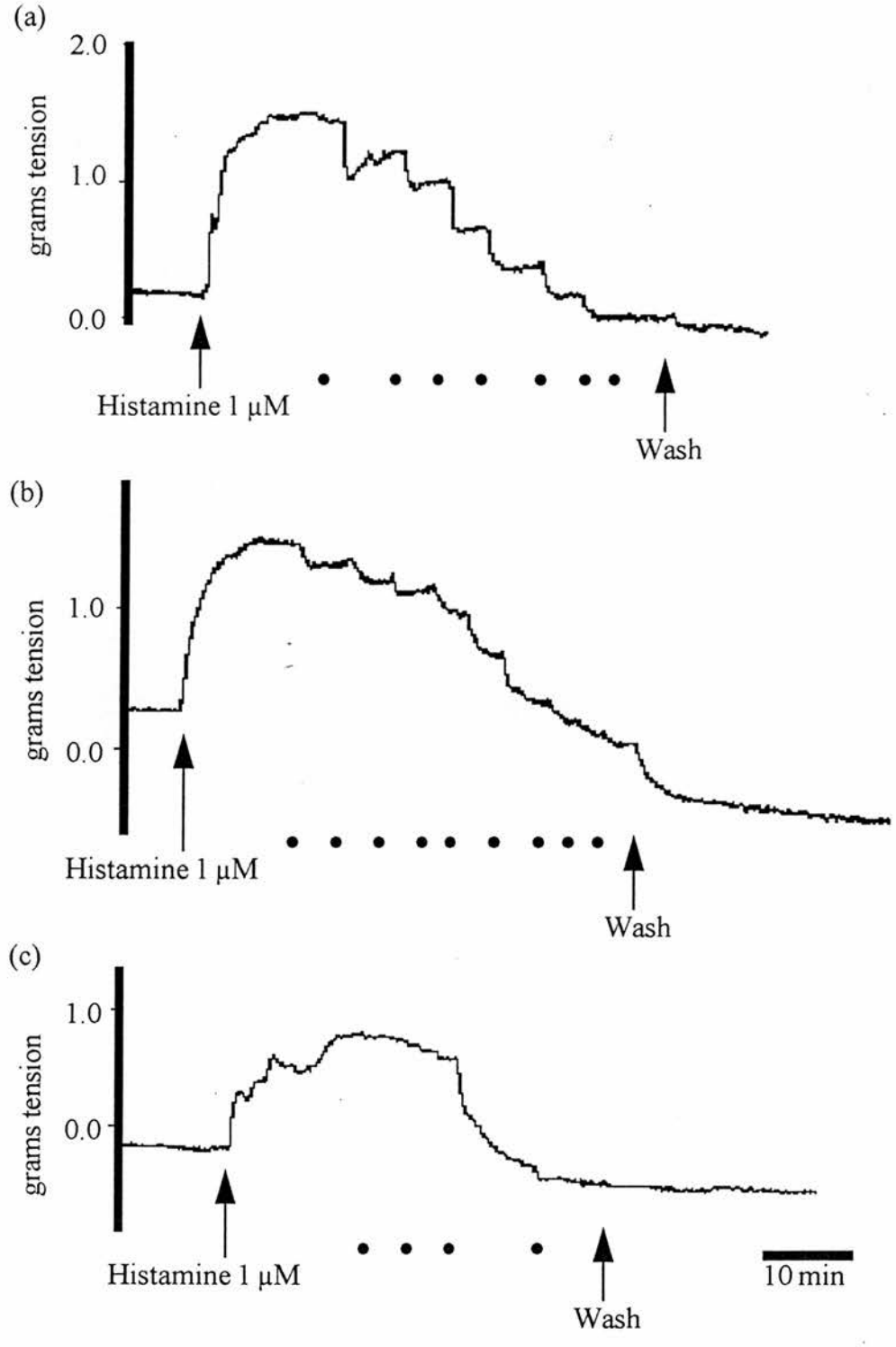

Figure 2.2.2- Examples of cumulative concentration-response curves in the RJV for (a) PGE_2 (0.1-100 nM), (b) 16,16 dimethyl PGE_2 (0.1 nM- 1 μM) and (c) AH13205 (1-30 μM). All concentration-response curves were carried out at 37°C in Krebs solution with GR32191X 10 μM and indomethacin 1 μM. •- addition of drug

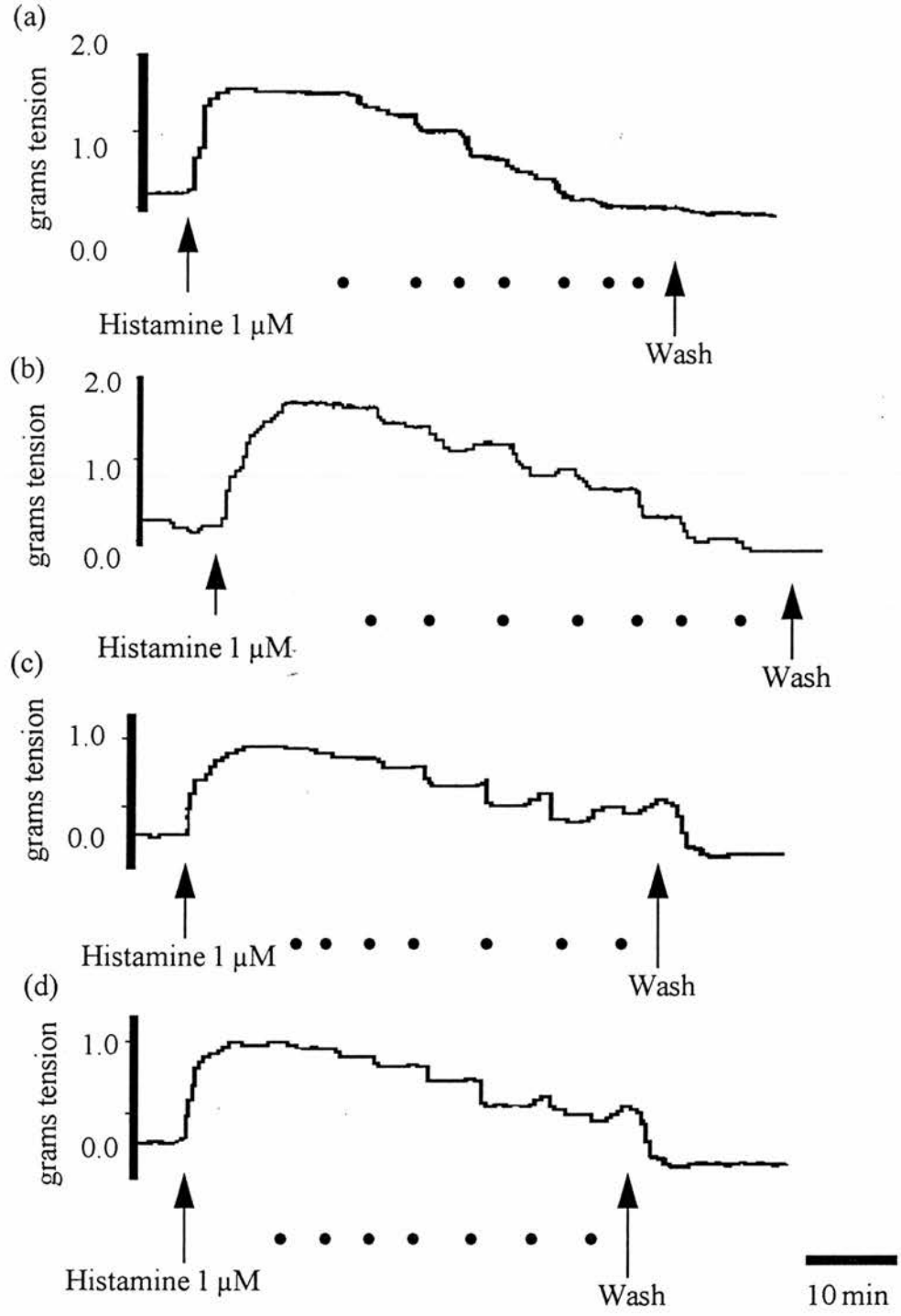

Figure 2.2.3- Examples of cumulative concentration-response curves in the RJV for (a) PGE_2 (0.1-100 nM), (b) PGE_2 (0.1-100 nM) + AH23848B (30 μM), (c) isoprenaline (1 nM- 1 μM) and (d) isoprenaline (1 nM- 1 μM) + AH23848B (30 μM). All concentration-response curves were carried out at 37°C in Krebs solution with GR32191X 10 μM and indomethacin 1 μM.
•- addition of drug

IC values

Concentration-response curves for PGE_2 were carried out at the start and finish of each experiment. PGE_2 was used as the control in all experiments and complete relaxation of the precontracted tissues by PGE_2 was taken as the maximal relaxation (100%). All subsequent results were expressed as percentages of the PGE_2 maximum. Log concentration-response curves were plotted and the IC_{50} values determined using Kaleidagraph® software. Each mean equi-effective concentration ratio (EEC) was calculated as the IC_{50} for the EP agonist/the IC_{50} for PGE_2.

Statistical tests

Results are presented as the mean ± SEM. Student's paired 1- or 2-tail *t*-tests were used for comparison of IC_{25} and IC_{50} data. ANOVA was used to compare concentration-response curves in the absence or presence of an antagonist.

2.2.3.- Results

Pig Saphenous Vein

PGE_2, the standard agonist, concentration-dependently inhibited phenylephrine-induced contractions of the PSV with an IC_{25} of 0.7 ± 0.4 nM and an IC_{50} of 2.0 ± 0.9 nM, Figure 2.2.4a. Pre-incubation with the EP_4 receptor antagonist AH23848B (30 μM, 30 min) shifted the concentration-response curve for PGE_2 to the right giving a CR of 5.94 ± 1.45 and an apparent pA_2 of 5.27, $p<0.05$ comparing IC_{50} values (paired two-tailed *t*-test), Figure 2.2.3a. Pre-incubation with AH6809 (10 μM, 10 min) had no significant effect on relaxant concentration-effect curves to PGE_2, with a CR of 2.95 ± 1.31, $p>0.05$, Figure 2.2.3b.

Comparison of the ability of various agonists, with activity at the EP_2 receptors, to relax the PSV gave a rank order of potency of $PGE_2 \geq$ 11-deoxy $PGE_1 \geq$ 16,16-dimethyl PGE_2 > butaprost >> AH13205, Figures 2.2.4a & b. All the agonists induced at least 95% of the maximum induced by PGE_2, IC_{50} values and EECs are shown in Table 2.2.1.

$PGF_{2\alpha}$ was also tested in this system, and concentration-dependently relaxed the PSV to 45.7 ± 6.6 % of the PGE_2 maximum giving an IC_{25} of 23.8 ± 7.3 nM and an EEC of 34 with respect to PGE_2, Figure 2.2.5.

Rabbit Jugular Vein

PGE_2 concentration-dependently inhibited histamine-induced contractions in the RJV with an IC_{25} of 2.08 ± 0.38 and an IC_{50} of 8.73 ± 1.90 nM, Figure 2.2.11a. Pre-incubation with the EP_4 antagonist AH23848B had no effect on PGE_2-induced relaxation (CR = 1.75 ± 0.67, Figures 2.2.3a & b, 2.2.7a). AH23848B also had no effect on relaxant responses to isoprenaline, butaprost and $PGF_{2\alpha}$ on the RJV, Figures 2.2.3c & d, 2.2.8a, b & c. Interestingly, AH23848B increased significantly the below maximum relaxation induced with 11-deoxy PGE_1 from 72.2 ± 8.4% to 88.1 ± 5.7%, Figure 2.2.6b

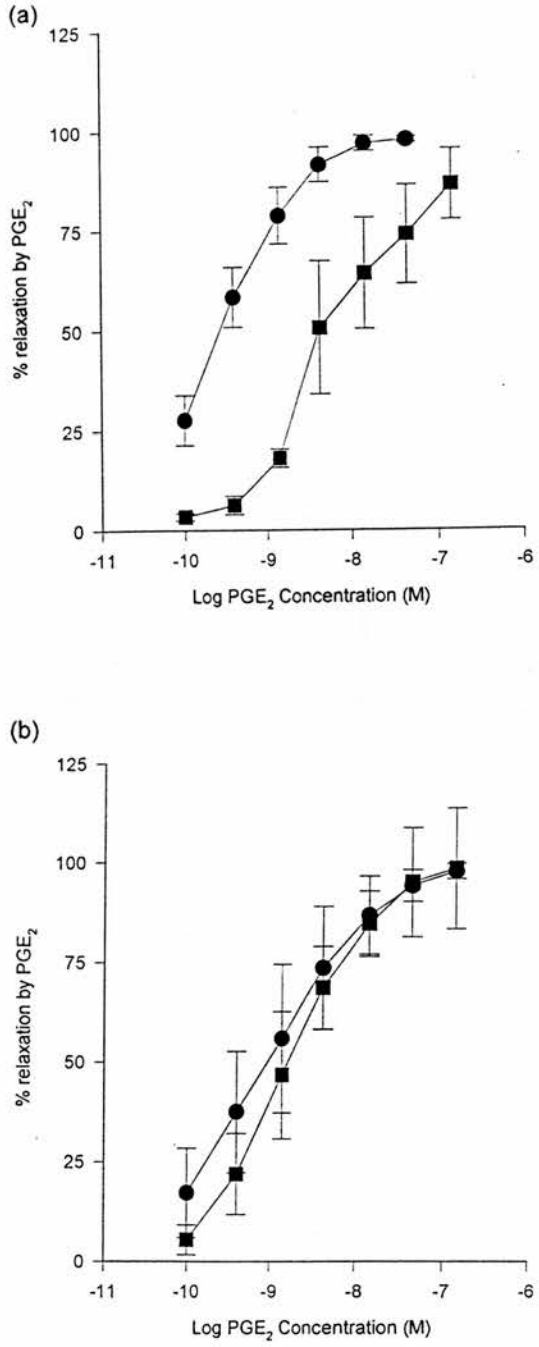

Figure 2.2.4.- (a) Log concentration-response curves on the PSV for PGE_2 alone (●), and in the presence of AH23848B 30 μM (■), n=4, $p<0.05$ with ANOVA. (b) Log concentration-response curves on the PSV for PGE_2 alone (●), and in the presence of AH6809 10 μM (■), n=3, $p>0.05$ with ANOVA. Relaxation in all curves reached >80% of the PGE_2 maximum.

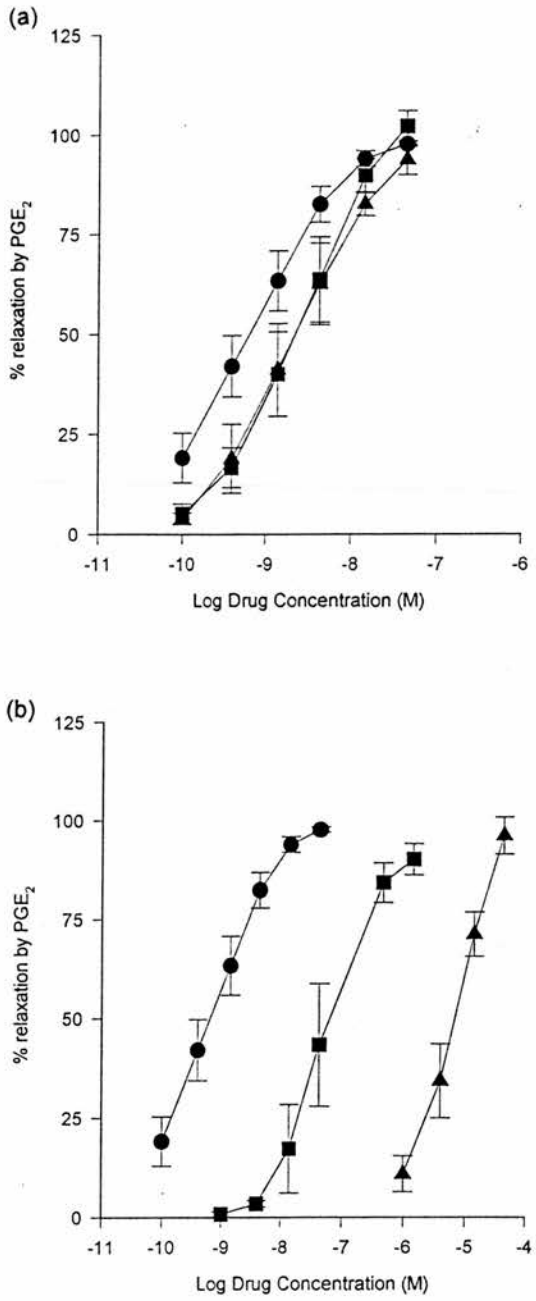

Figure 2.2.5.- (a) Log concentration-response curves on the PSV for PGE_2 (●), n=7, 16,16-dimethyl PGE_2 (■), n=3, and 11-deoxy PGE_1 (▲), n=5, and (b) for PGE_2 (●), n=7, butaprost (■), n=4, and AH13205 (▲), n=4. Relaxation for all compounds reached >80% of the PGE_2 maximum.

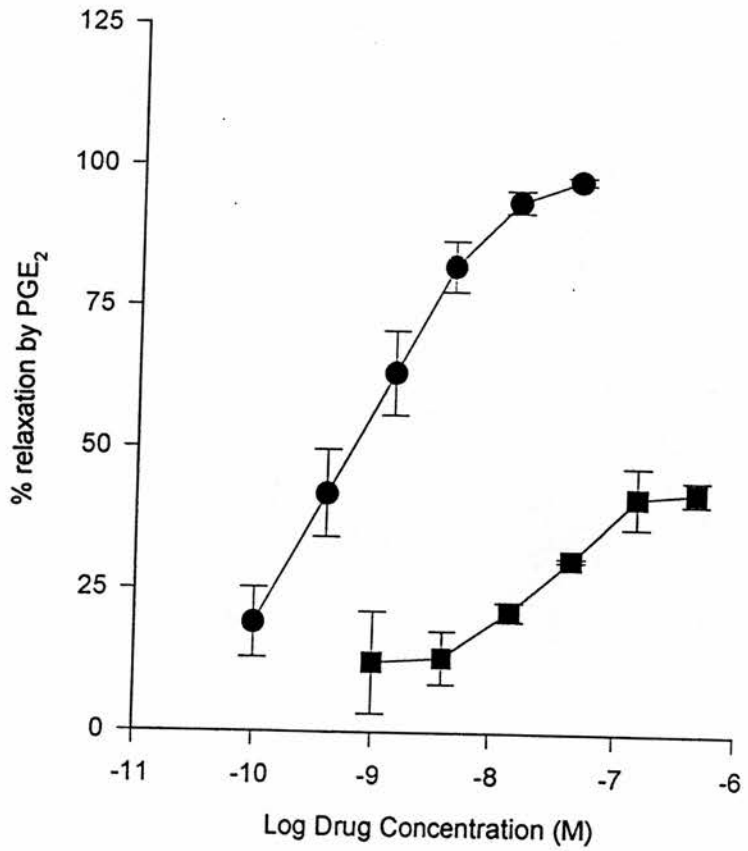

Figure 2.2.6.- Log concentration-response curves on the PSV for PGE_2 (●), n=7, and $PGF_{2\alpha}$ (■), n=3. $PGF_{2\alpha}$ achieved a maximum relaxation of 45.7 ± 6.6% compared to PGE_2.

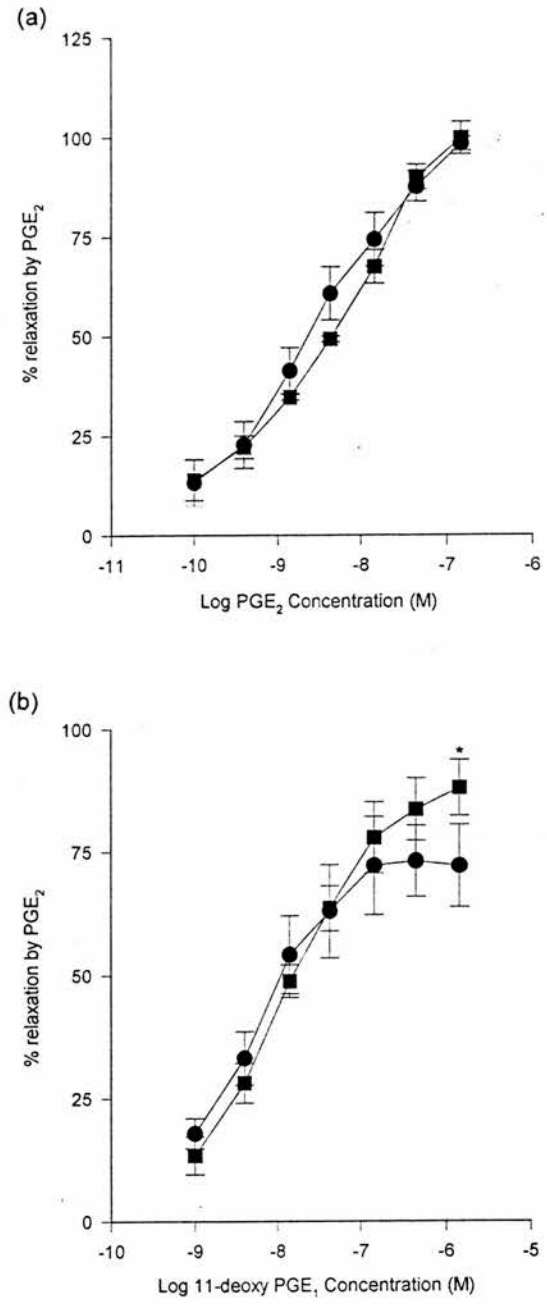

Figure 2.2.7.- (a) Log concentration-response curves on the RJV for PGE_2 alone (●), and in the presence of AH23848B 30 μM (■), n=4, and (b) for 11-deoxy PGE_1 alone (●), and in the presence of AH23848B 30 μM (■), n=4. AH23848B had no effect on the concentration-response curves for both compounds and $p>0.05$ with ANOVA for both graphs.
*- $p<0.05$, paired two-tailed Student *t*-test.

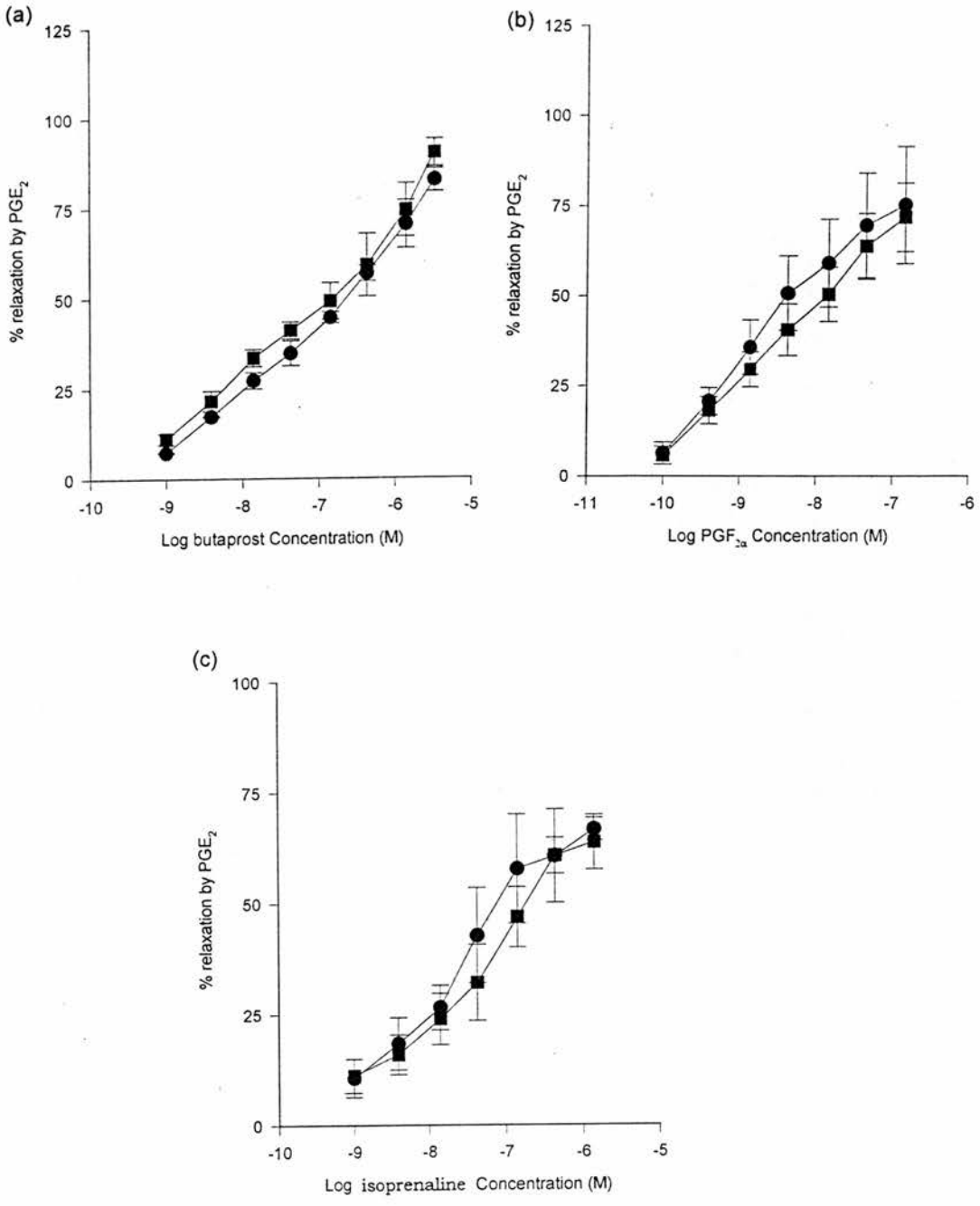

Figure 2.2.8.- (a) Log concentration-response curves on the RJV for butaprost alone (●), and in the presence of AH23848B 30 μM (■), n=4, (b) for $PGF_{2\alpha}$ alone (●), and in the presence of AH23848B 30 μM (■), n=6, and (c) for isoprenaline alone (●), and in the presence of AH23848B 30 μM (■), n=4. AH23848B had no effect on the concentration-response curves for any of the compounds, $p>0.05$ with ANOVA in all experiments.

Pre-incubation with AH6809 (10 μM, 10 min) and BW A868C (5.4 μM, 10 min) also had no significant effects on the PGE_2 concentration-response curve, producing CRs of 0.78 ± 0.33 and 1.75 ± 0.84 respectively, Figures 2.2.8a & c. AH6809 was also examined against $PGF_{2\alpha}$ induced relaxation of the RJV and produced no effect, Figure 2.2.8b.

L-NMMA was examined in this preparation to measure the dependency of an intact endothelial layer. Used at a concentration of 100 μM (15 min pre-incubation), L-NMMA had no significant effect on PGE_2, 16,16-dimethyl PGE_2, $PGF_{2\alpha}$ and 17-phenyl trinor $PGF_{2\alpha}$ induced relaxation of the RJV, Figures 2.2.9a & b, & 2.2.10a & b.

A range of EP receptor agonists were examined for their relaxant effects on the RJV. The rank order of potency obtained for these agonists was $PGE_2 \geq$ nocloprost > 11-deoxy PGE_1 > 16,16 dimethyl PGE_2 > butaprost >> AH13205, Figures 2.2.11a & b, Table 2.2.1. All these agonists, except 11-deoxy PGE_1, produced a maximal relaxation equivalent to at least 90% of that to PGE_2, 11-deoxy PGE_1 only achieved a maximal relaxation of 73.1 ± 7.2%, Figure 2.2.11a.

The naturally-occurring prostaglandins, PGD_2 and $PGF_{2\alpha}$, were examined in this preparation along with the FP selective agonist, 17-phenyl trinor $PGF_{2\alpha}$. All three compounds produced relaxation of the RJV giving EECs of 13.5 ± 11.2, 6.1 ± 3.0 and 25.4 ± 16.6 respectively, however, only $PGF_{2\alpha}$ produced greater than 60 % relaxation compared to the maximum response for PGE_2, Figure 2.2.12a.

Agonist data in Table 2.2.1 confirm the relatively low potency of butaprost on the RJV (Lawrence & Jones, 1992). However, although the IC_{50} values for butaprost are similar in the two studies, 381.0 ± 240.5 nM here compared to 199.5 nM, the EEC values of 43 and 685 are different and reflect the potency of PGE_2 in the two studies (IC_{50}s of 8.73 ± 1.9 nM and 0.46 nM, respectively), Table 2.2.2. The reasons for the difference in sensitivity of the RJV to PGE_2 in these two studies are still not entirely clear, but there are two possibilities for the

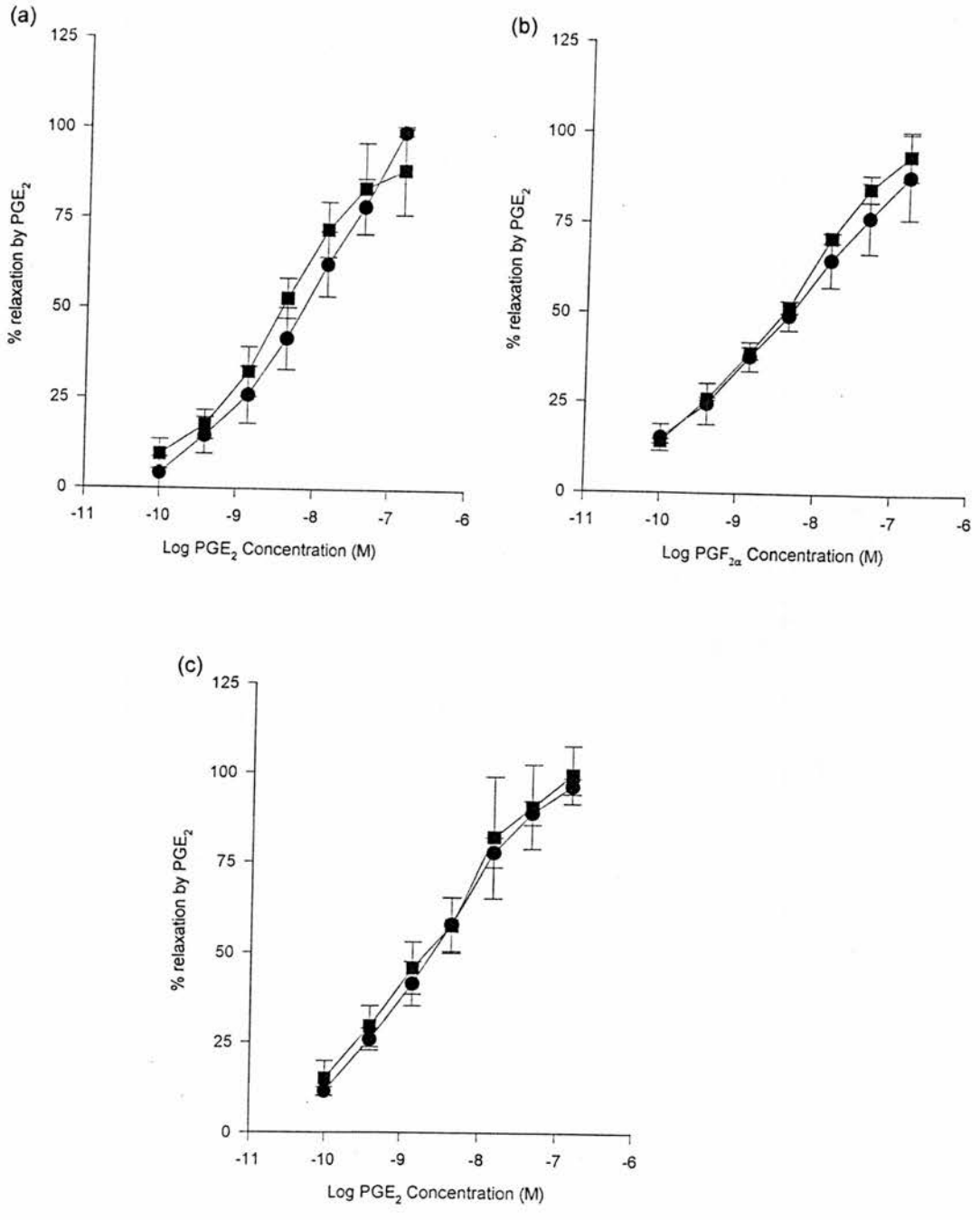

Figure 2.2.9.- (a) Log concentration-response curves on the RJV for PGE_2 alone (●), and in the presence of AH6809 10 μM (■), n=4, and (b) for $PGF_{2\alpha}$ alone (●), and in the presence of AH6809 10 μM (■), n=3. AH6809 had no effect on the concentration-response curves for both compounds, $p>0.05$ in both experiments. (c) Log concentration-response curves on the RJV for PGE_2 alone (●), and in the presence of BW A868C 5.4 μM (■), n=3. BW A868C had no effect on the concentration-response curves for PGE_2, $p>0.05$ with ANOVA.

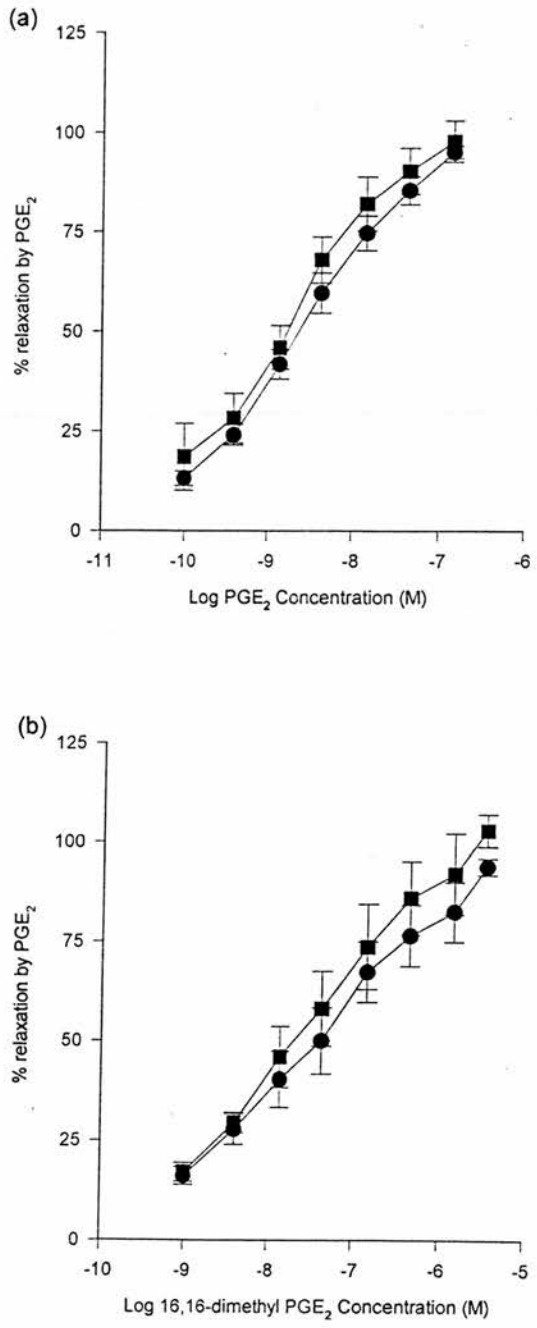

Figure 2.2.10. - Log concentration-response curves on the RJV for PGE_2 alone (●), and in the presence of L-NMMA 100 μM (■), n=8, and (b) for 16,16-dimethyl PGE_2 alone (●), and in the presence of L-NMMA 100 μM (■), n=5. L-NMMA had no effect on the concentration-response curves for both compounds, $p>0.05$ with ANOVA in both experiments.

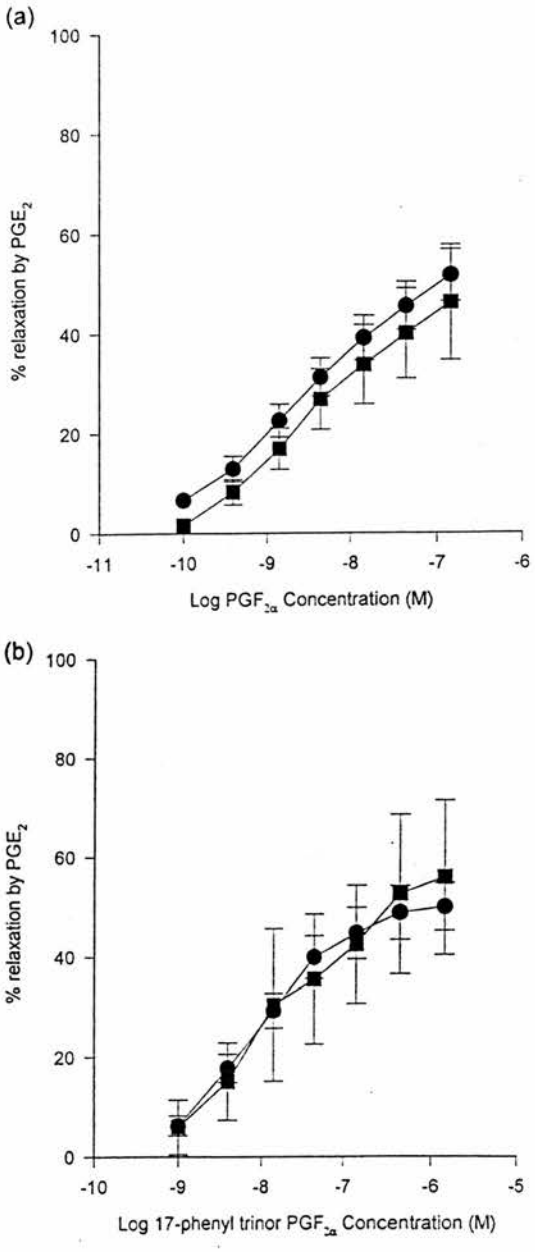

Figure 2.2.11.- (a) Log concentration-response curves on the RJV for $PGF_{2\alpha}$ alone (●), and in the presence of L-NMMA 100 μM (■), n=6, and (b) for 17-phenyl trinor $PGF_{2\alpha}$ alone (●), and in the presence of L-NMMA 100 μM (■), n=3. L-NMMA had no effect on the concentration-response curves for both compounds, $p>0.05$ with ANOVA in both experiments.

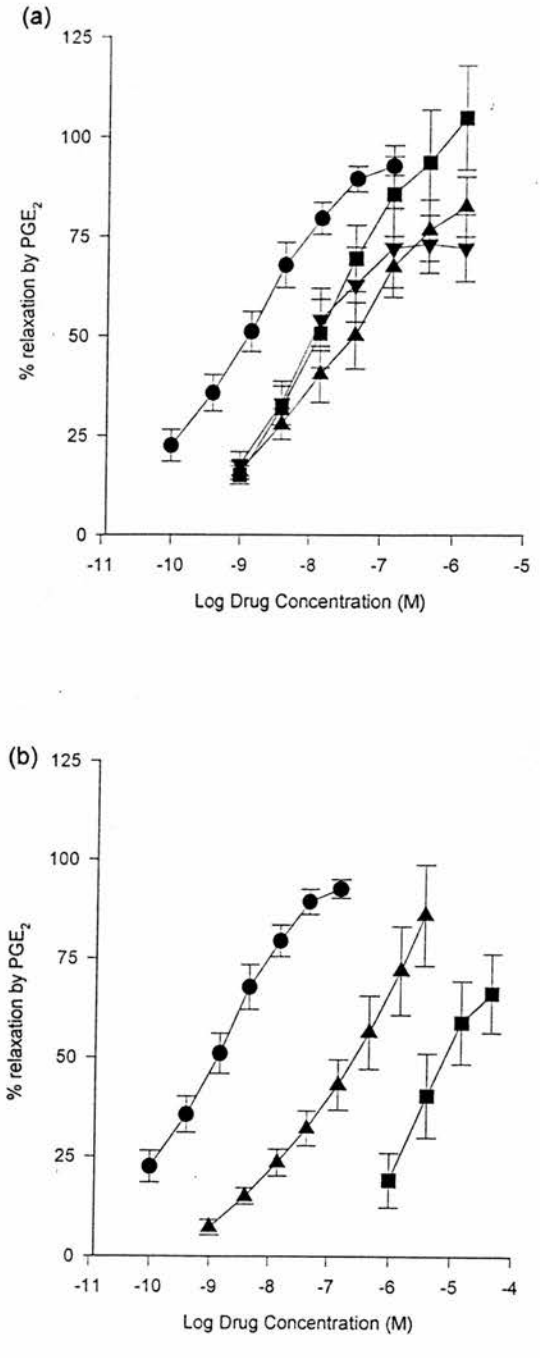

Figure 2.2.12.- (a) Log concentration-response curves on the RJV for PGE_2 (●), n=10, nocloprost (■), n=4, 16,16-dimethyl PGE_2 (▲), n=5, and 11-deoxy PGE_1 (▼), n=4, and (b) PGE_2 (●), n=10, AH13205 (■), n=5, and butaprost (▲), n=4. Relaxation for all compounds reached >80% of the PGE_2 maximum, except 11-deoxy PGE_1 which only produced 73.1 ± 7.2%.

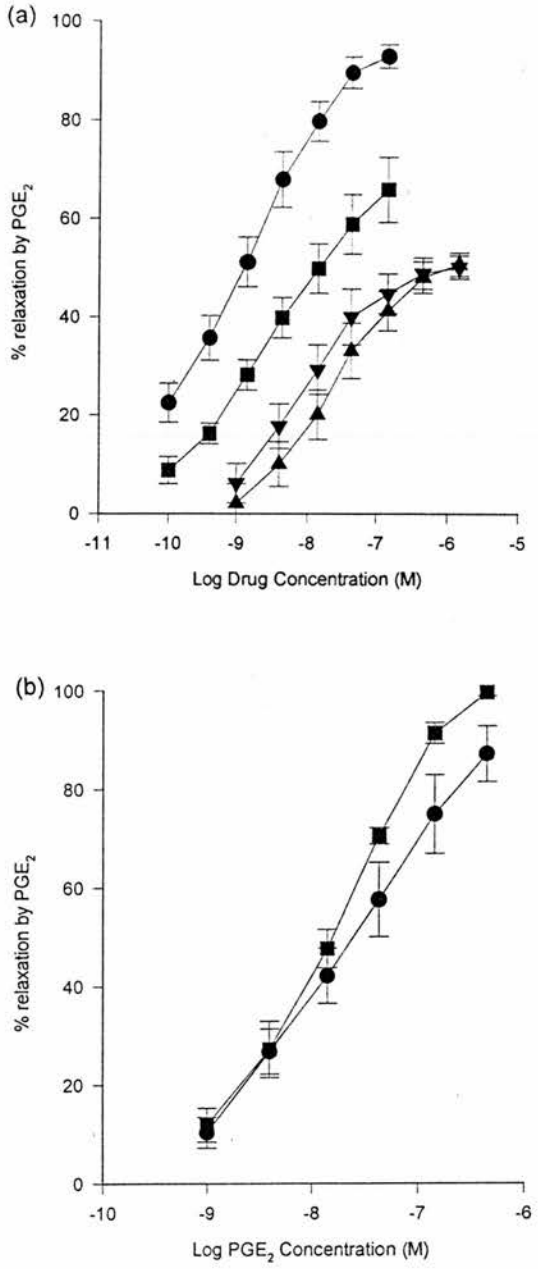

Figure 2.2.13.- (a) Log concentration-response curves on the RJV for PGE_2 (●), n=10, and $PGF_{2\alpha}$ (■), n=14, 17-phenyl trinor $PGF_{2\alpha}$ (▼), n=4, and PGD_2 (▲), n=3. Maximal relaxation compared to PGE_2 are 65.7 ± 6.3%, 50.0 ± 4.7% and 50.6 ± 4.4% respectively. (b) Effect of high concentration (5 μM) (●), and low concentration (2 μM) (■) histamine on the PGE_2 dose-response curve in the RJV.

*- $p<0.05$ paired two-tailed Student *t*-test

Agonist	IC_{50} PSV	RJV
PGE_2	2.05 ± 0.93 nM	8.73 ± 1.90 nM
nocloprost		14.87 ± 3.13 nM
11-deoxy PGE_1	3.17 ± 1.41 nM	61.32 ± 52.91 nM
16,16 dimethyl PGE_2	3.37 ± 1.23 nM	87.03 ± 34.18 nM
butaprost	83.9 ± 21.8 nM	381.0 ± 240.5 nM
AH13205	6.36 ± 2.58 μM	20.26 ± 8.30 μM

Table 2.2.1- IC_{50} values for a range of EP agonists in the PSV and RJV

differences and taken together these may account for the apparent loss of sensitivity in our preparation.

One possibility is the concentration of histamine used to precontract the RJV. While submaximally-effective concentrations were used in our studies, usually 2-5 μM, Lawrence and Jones (1992) typically used 1 μM. Relaxation by PGE_2 of the histamine-induced contractions is a form of functional antagonism, so perhaps a smaller contraction is easier to block than a larger contraction. This can be seen to a limited extent in Figure 2.2.12b, where significantly more relaxation was observed with PGE_2 0.14 μM and 0.45 μM using 2 μM compared with 5 μM histamine ($p<0.05$).

Secondly, definition of the maximal relaxation induced by PGE_2 is different in the two studies. Lawrence and Jones, 1992, used the contractile response to histamine as 100%, whereas we have used the complete relaxation induced by PGE_2 as 100%. Since the tissues are already under tension before the addition of histamine, PGE_2 usually relaxes the tissue to a level below the basal tension prior to the addition of histamine (see Figure 2.2.2). Calculating the IC_{50} value for PGE_2 by these two methods in the same preparations gives values of 7.9 ± 2.4 nM using the observed relaxation induced by PGE_2 as the maximum, and 3.8 ± 1.5 nM when relaxation of the precontraction induced by histamine is taken as the maximum.

While these points may partly explain the lower sensitivity to PGE_2 in our experiments compared to those of Lawrence and Jones, 1992, this should not affect the EEC values calculated in both studies and so does not explain the greater sensitivity to butaprost seen here. The EECs of 6.6 and 9.9 for 11-deoxy PGE_1 and 16,16-dimethyl PGE_2 found in this study, Figure 1.1.2, compare more favourably with those of 1.4 and 2.1 found by Lawrence and Jones (1992).

2.2.4.- Discussion

The purpose of these experiments was to further characterise the EP receptor subtype present in the PSV with a range of EP receptor agonists and to characterise the relaxant EP receptor(s) present in the RJV. Ideally, the determination of the specificity of an agonist requires an estimation of both the affinity and efficacy of the compound at a particular receptor. The most reliable method for determining these variables involves irreversible receptor blockade (Furchgott, 1966), but unfortunately no irreversible antagonists have been described yet at EP receptor subtypes. In this study the comparison of the relative potencies of EP agonists, and possible block by receptor antagonists where available, have been used in an attempt to characterise the receptor subtypes involved.

The PSV has been confirmed to contain EP_4 receptors, with the antagonist AH23848B, Figure 2.2.4a, giving an apparent pA_2 of 5.3. This agrees favourably with that previously obtained this preparation by Louttit *et al.*, (1992a) and Coleman *et. al.*, (1994), and on the cloned EP_4 receptor by Nishigaki *et al.*, (1995). As only one concentration of AH23848B was tested, the pA_2 value for AH23848B can only be estimated with the assumption that it would generate a Schild plot -log (antagonist concentration) against log (concentration ratio-1) with a gradient of 1. It is unlikely that a component of the relaxation observed with PGE_2 is mediated by DP receptors as AH6809 did not affect the concentration-response curve for PGE_2, Figure 2.2.4b. Furthermore, Coleman *et al.*, 1994, demonstrated the rank order of potency for the natural prostanoids and the synthetic TxA_2 mimetic, U-46619, at relaxing the PSV was $PGE_2 >> PGI_2 \sim PGF_{2\alpha} \geq PGD_2 > U\text{-}46619$, which shows, by definition, that only EP receptors are involved in mediating relaxation of the PSV.

The IC_{50} determined for PGE_2 on this preparation is 2.05 ± 0.93 nM, which agrees well with the value of 2.5 nM determined by Coleman *et al.*, 1994. 11-deoxy PGE_1, 16,16-dimethyl PGE_2, butaprost and AH13205 all produced full relaxation of the PSV with relative activities of $PGE_2 \geq$ 11-deoxy PGE_1 = 16,16-dimethyl PGE_2 > butaprost >> AH13205, Figure 2.2.5a & b. From these results it can be suggested

that 11-deoxy PGE_1 and 16,16-dimethyl PGE_2 are putative agonists at the EP_4 receptor, with EEC values relative to PGE_2 of 2 and 2.8 respectively, Table 2.2.1. This has now been confirmed with work on the cloned EP_4 receptor where these two compounds both displaced membrane-bound $[^3H]PGE_2$ with identical K_is of 7.3 relative to PGE_2. 11-deoxy PGE_1 was also tested for its ability to increase cAMP accumulation in intact transfected CHO cells and produced an EEC of 0.75 relative to PGE_2 (Nishigaki *et al.*, 1995).

The RJV is known to contain a heterogeneous population of prostanoid receptors, namely thromboxane-sensitive (TP) receptors mediating contraction (blocked here with the TP receptor antagonist GR32191X) and receptors for PGE_2, PGD_2, and PGI_2 mediating relaxation (Giles *et. al.*, 1990; Leff & Giles, 1992; Lawrence & Jones, 1992).

The EP_4 antagonist AH23848B (Louttit *et al.*, 1992b) was examined in this preparation and had no effect against PGE_2, butaprost or $PGF_{2\alpha}$ which suggests that EP_4 receptors are not involved in mediating the relaxation properties of these compounds, Figures 2.2.7a, 2.2.8a & b. AH6809 and BW A868C were also studied but did not block the relaxant response of PGE_2. Similarly, AH6809 did not block $PGF_{2\alpha}$, Figures 2.2.9a-c, indicating that PGE_2 and $PGF_{2\alpha}$ induced relaxation are not mediated by DP receptors. Furthermore, the relaxant activity of PGD_2 compared with PGE_2 and $PGF_{2\alpha}$ illustrates the limited relaxation obtained with DP receptor agonists in this preparation, Figure 2.2.13a.

The NO synthase L-NMMA did not significantly affect relaxation induced by PGE_2, 16,16-dimethyl PGE_2, $PGF_{2\alpha}$ or 17-phenyl $PGF_{2\alpha}$, Figure 2.2.10a & b, & 2.2.11a & b, indicating that the receptor mediating relaxation by these compounds is present on the smooth muscle and is not endothelium-dependent.

Agonist studies on the RJV demonstrated that 11-deoxy PGE_1 and 16,16-dimethyl PGE_2 relaxed the RJV with EEC values of 6.6 and 9.9, Figures 2.2.12a, Table 2.2.1, which are similar to their activities on the cat trachea, an EP_2-receptor containing preparation, where they

produced EEC values of 13 and 9.4 (Dong *et al.*, 1986). Butaprost and AH13205, selective EP_2-agonists, however, gave much lower EEC values of 43 and 2780 respectively which do not compare as favourably with those obtained for the two compounds in cat trachea and rabbit ear artery, EP_2 receptor containing preparations. Butaprost has an EEC of 6-30 in these preparations and AH13205 an EEC of 30-100 (Gardiner, 1986; Humbles *et al.*, 1991; Nials *et al.*, 1993). None of the above data define any particular EP receptor system in the RJV, nevertheless, the ability of a range of EP receptor agonists to completely relax histamine contraction in the RJV and the inactivity of selective antagonists leaves the RJV still classified as an atypical EP_2-receptor. Further to this is a recent publication which demonstrates that AH6809 has antagonist activity at the recombinant EP_2 receptor (Woodward *et al.*, 1995). AH6809 10 μM had no effect in this system and provides further evidence to classify this system 'atypical EP_2'.

Interestingly, comparison of the agonist profiles for the PSV and RJV could suggest that the RJV is a preparation containing EP_4 receptors at which the EP_4 antagonist AH23848B is less potent, Table 2.2.2. If this was the case there could be a few reasons for explaining the lack of AH23848B activity. It has been documented elsewhere that TP antagonists have lower affinity for TP receptors in the rabbit than for other TP receptor-containing preparations (Tymkewycz *et. al.*, 1991), and this may be true here for EP receptors, with AH23848B interestingly originally being described as a TP antagonist (Brittain *et al.*, 1985). Unfortunately, it is difficult to test AH23848B at concentrations above 30 μM in the organ bath due to its insolubility and experiments with the more recently described EP_4 receptor antagonist AH22921 (Coleman *et. al.*, 1994) might help answer this question. However, against this possibility is the demonstration that AH23848B is active in the rabbit and has been shown to inhibit PGE_2 induced relaxation of indomethacin and KCl precontracted rabbit ductus arteriosus with a pA_2 of 4.9 (Smith *et al.*, 1995).

Agonist	EEC	
	PSV	RJV
PGE_2	1.0	1.0
nocloprost		1.7
11-deoxy PGE_1	2.0	6.6
16,16 dimethyl PGE_2	2.8	9.9
butaprost	42	43
AH13205	3100	2780

Table 2.2.2- EEC values for a range of EP agonists in the PSV and RJV

The possibility that antagonism with AH23848B was masked by an additional action of AH23848B to potentiate the relaxant effect of PGE_2 was investigated. Such potentiation has been observed in human neutrophils using both PGE_2 and an adenosine analogue as agonists (Talpain *et al.*, 1995). However relaxation of the RJV by isoprenaline (see Figures 2.2.3c & d, Figure 2.2.8c) was unaffected by AH23848B (30 μM) suggesting that this explanation is unlikely to account for the lack of antagonism observed.

The exclusion of interaction at IP receptors has not been addressed here as there are no selective IP antagonists available, but this mechanism seems unlikely, as PGE_2 is more potent on the RJV than are PGI_2 or selective IP agonists (Giles *et. al.*, 1990, Lawrence & Jones, 1992).

The possible existence of a novel relaxant FP receptor was also investigated in this study. $PGF_{2\alpha}$ and 17-phenyl $PGF_{2\alpha}$ both relaxed the RJV, Figures 2.2.13a. $PGF_{2\alpha}$-induced relaxation was not inhibited by AH23848B or AH6809, Figures 2.2.8b & 2.2.9b, and L-NMMA failed to inhibit either agonist suggesting the receptor(s) are present on the smooth muscle, Figures 2.2.11a & b. From this it is difficult to exclude the presence of a novel receptor since these drugs only rule out EP_4-receptors, EP_1/DP-receptors and endothelium dependency respectively. Also, the EEC of $PGF_{2\alpha}$ with respect to PGE_2 is only 6.1 which, by prostanoid receptor classification, is too close to exclude presence of FP receptors, albeit mediating a relaxant response. $PGF_{2\alpha}$ produced only partial relaxation of the PSV with a similar EEC of 11.9 compared with 6.1 in the RJV, Figure 2.2.6. Unfortunately, the effects of AH23848B on $PGF_{2\alpha}$-induced relaxation in the PSV were not examined in this study to see if $PGF_{2\alpha}$ could be mediating relaxation via EP_4 receptor activation. Agonism of EP_3 receptors positively coupled to AC was excluded in the RJV by Lawrence & Jones as sulprostone generated an EEC of >3000 relative to PGE_2 (Lawrence & Jones, 1992). To determine if the RJV contains a novel FP receptor or whether FP is acting via EP_4 receptor activity, a more potent EP_4-antagonist will need to be examined.

The ability of FP agonists to lower IOP has a few possible explanations. The results by Woodward and co-workers indicating a negative correlation between FP selectivity in agonists and their ability to lower IOP led to the conclusion that FP receptors are not involved in this system (Woodward *et al.*, 1993). However, a later study by the same group showed the presence of FP receptors through binding studies (Woodward *et al.*, 1994). Possible pathways in both IOP reduction and hyperemia might be the involvement of desensitised FP receptors which couple to AC, or an EP receptor for which $PGF_{2\alpha}$ has high affinity. The latter of these may be the case as $PGF_{2\alpha}$ induces greater relaxation in the RJV than the more selective FP agonist 17-phenyl trinor $PGF_{2\alpha}$ and has a lower EEC relative to PGE_2. However, further studies are required to identify how $PGF_{2\alpha}$ and other analogues mediate their actions.

It is interesting that the cloning of the EP receptors has been based functionally on the activities of certain selective agonists and antagonists. Butaprost has been shown to exhibit no activity at the cloned EP_4 receptor either functionally or at displacing radioactive ligand (Honda *et al.*, 1993; Nishigaki *et al.*, 1995), whereas it exhibits both functional and binding activity at the cloned EP_2 receptor (Regan *et al.*, 1994). Of interest here is the ability of butaprost to induce complete relaxation of the RJV and PSV, as does AH13205, another selective EP_2 agonist. If the cloning data are correct, and butaprost has no activity at EP_4 receptors, this suggests that there must be co-expression of EP_2 and EP_4 receptors in both the PSV and the RJV. The reason that AH23848B is not active in the RJV, may relate to differential expression of the EP_2 and EP_4 receptors subtypes. Block of a small EP_4 component may not be identified, because PGE_2 could still induce complete relaxation at EP_2 receptors. This explanation would however require a different proportion of EP_2/EP_4 receptor subtypes in the RJV and PSV preparation to account for the differential antagonism observed with AH23848B only in the PSV.

Interestingly, the maximum response observed with 11-deoxy PGE_1 in the RJV was increased in the presence of AH23848B. The co-expression of EP_2 and EP_4 receptors may explain this result. If 11-deoxy PGE_1 is a partial agonist at the EP_4 receptor (this is supported

by observations in the CHO cell line where it produced a submaximal response relative to PGE_2) but a full agonist at the EP_2 receptor, then, 11-deoxy PGE_1 may possess higher affinity for the EP_4 rather than the EP_2 receptor. Block of the EP_4 receptor with AH23848B may then favour the binding of 11-deoxy PGE_1 to the EP_2 receptor where it might induce complete relaxation of the RJV.

It is interesting to note that in the cloned EP_4 receptor 11-deoxy PGE_1 completely displaces [^{3}H]PGE_2 but only produces about 70% of the maximal cAMP generation seen with PGE_2 with an EEC of 0.75, suggesting that it is a partial agonist at this receptor (Nishigaki *et al.*, 1995). Data from the cloned EP_2 receptor, however, demonstrates that 11-deoxy PGE_1 only produces about 80% displacement of [^{3}H]PGE_2 and it is not clear whether 11-deoxy PGE_1 is a full or partial agonist at this receptor (Regan *et al.*, 1994). Calculating the K_i values for 11-deoxy PGE_1 at the EP_2 and EP_4 receptor, using the K_i and EEC values of Nishigaki and co-workers (1995), gives 100.7 nM and 58.4 nM respectively showing that 11-deoxy PGE_1 has a higher affinity for the EP_4 receptor. The existence of both receptors in the RJV may account for these results with 11-deoxy PGE_1.

In conclusion, the PSV has been confirmed to be an EP_4 receptor containing preparation and 11-deoxy PGE_1 and 16,16 dimethyl PGE_2 have been shown to have agonist activity at the EP_4 receptor subtype. The RJV, however, remains an atypical EP_2 receptor containing preparation due to the inactivity of AH23848B and the high EEC values for butaprost and AH13205 with respect to PGE_2. Therefore, further studies are required with the RJV before a system can be defined in which anti-glaucoma drugs can be screened, and the RJV, although used, remains uncharacterised for relaxant EP receptors. A possible explanation for the observed results with butaprost, AH13205, 11-deoxy PGE_1 and AH23848B may be that both the PSV and the RJV co-express the EP_2 and EP_4 receptor subtypes.

2.3.- Conclusion

Comparison of data obtained for all the currently described EP_4 receptor containing preparations, Table 2.3.1, show that in most cases the 50% active concentration for PGE_2 is less than 20 nM, and that 11-deoxy PGE_1 and 16,16-dimethyl PGE_2 are equipotent at all EP_4 and EP_2 receptor containing preparations.

Interestingly, it is only in the CHO cell that PGE_2 has an EC_{50} of greater than 20 nM and 11-deoxy PGE1 and 16,16 dimethyl PGE_2 have EEC values of greater than 100. Also, it is only in the native CHO cell receptor that AH23848B has a pA_2 of greater than 6.0. As discussed previously, these differences may be due to a low receptor expression and a sensitive system which is easily inhibited. Nevertheless, these results may also show that these cells only express EP_4 receptors, with little or no expression of the EP_2 subtype. Another study with the Jurkat T-cell line demonstrates no agonist activity for butaprost and AH13205 with AH23848B acting as a non-competitive antagonist (De Vries *et al.*, 1995). The group suggests that the EP receptor has a pharmacological profile different from the other EP subtypes and suggest it might be a variant of the EP_4 receptor.

Compared to the cloned receptor, it is only in these cell line preparations that butaprost and AH13205 demonstrate no agonist activity. It could be suggested that the two cell lines, CHO and Jurkat, express only EP_4 receptors and that the smooth muscle preparations co-express EP_2 and EP_4 receptors.

In the cloned EP_4 and native CHO cell line butaprost produces no accumulation of cAMP (Nishigaki *et al.*, 1995; Chapter 2.1), whereas in the PSV it has an EEC only just larger than its EEC at the EP_2 receptor, Table 2.3.1. As discussed previously, this agonist data may not reflect the cross-reactivity of butaprost but instead result from co-expression of EP_2 and EP_4 receptors in smooth muscle preparations.

Compound	PSV[1]	RIT[2]	RDA[3]	Jurkat cell[4]	Native CHO cell[5]	Cloned EP_4[6]	RJV[7]	EP_2 receptor[8]
	EC/IC_{50} nM							
PGE_2	2.05	15.4	0.36	80	94	20*	8.73	80
	EEC (PGE_2=1)							
11-deoxy PGE_1	2.0	2.4	n.t.	1.7-4.3	134	7.3	2.1-6.6	13
16,16 dimethyl PGE_2	2.8	2.8	n.t.	49-251	163	7.3	2.1-9.9	9.4-20
butaprost	42	n.t.	n.t.	>1500	>10,000	>1200	43-685	17
AH13205	3100-11,000	1120	>100,000	>1500	>10,000	n.t.	2780	29
	pA_2							
AH23848B	5.0-5.4	5.1-5.2	4.9		6.2	5.3	>4.5	>4.5

Table 2.3.1. - Comparison of EC/IC_{50} values for PGE_2, EEC and pA_2 values for EP receptor agonists and the EP_4 antagonist AH23848 in preparations reported to contain EP_4 receptors, the RJV and an EP_2 receptor preparation.

n.t.-not tested

1-(Chapter 2.2; Louttit *et al.*, 1992a,b; Coleman *et al.*, 1994)

2-Rat isolated trachea (Lydford & McKechnie, 1994)

3-Rabbit ductus arteriosus (Smith *et al.*, 1995)

4-Jurkat T-cell line(Woodward *et al.*, 1995)

*-K_i

5-(Chapter 2.1)

6-(Nishigaki *et al.*, 1995)

7-(Chapter 2.2; Lawrence & Jones, 1992)

8-Cat trachea (Dong *et al.*, 1986; Gardiner, 1986; Coleman *et al.*, 1988; Nials *et al.*, 1993)

The antagonism observed with AH13205 in the RDA (Smith *et al.*, 1994) may also be the result of receptor co-expression. Smith and co-workers demonstrated that both AH23848B and AH13205, at lower concentrations than those which relaxed the RDA, caused rightward shifts in the concentration-response curves to PGE_2 and BW245C, a DP agonist. It could be supposed that each compound is inducing these agonists to preferentially bind at the other EP receptor subtype. However it must be notesd that while butaprost has been shown to have no affinity for the cloned EP_4 receptor, AH13205 has not been tested and so at this stage it is not clear if AH13205 acts as a partial agonist at EP_4 receptors. This could be resolved with ligand binding experiments using cells expressing the cloned EP_4 receptor and AH13205 as the displacing agent. It is interesting to note that the presence of AH23848B produces a slight flattening of the relaxation curve to PGE_2 in the RDA, whereas, AH13205 induces no shape change. To determine if this is a real effect the efficacy of PGE_2 at each receptor needs to be measured.

The key experiment required to answer whether there are both EP_2 and EP_4 receptors expressed in smooth muscle preparations, would be to examine butaprost in the presence of AH23848B in the PSV where AH23848B has been shown to block PGE_2. Since butaprost is a full agonist in the PSV, if no shift in the concentration-response curve to butaprost is induced by AH23848B then the PSV must express both EP_2 and EP_4 receptors, verifying that butaprost is a selective EP_2 agonist. Whereas, if antagonism was observed with AH23848B it could be suggested that the PSV contained only EP_4 receptors and that butaprost was also an EP_4 agonist. This, however, would bring into doubt the results observed with the cloned receptors, since it was the inactivity of butaprost which originally identified the cloned EP_4 receptor (Honda *et al.*, 1993), and its activity which aided classification of the cloned EP_2 receptor (Regan *et al.*, 1994). Antagonism of the concentration-response curve to butaprost would suggest that the cloned receptors were another EP receptor subtype, possibly EP_5.

Clearly it is important to resolve this issue of co-expression of EP_2 and EP_4 receptors and to ascertain the selectivity of both butaprost and AH13205 as EP_2 receptor agonists. Further work using Northern blots and probes for the EP_2 and EP_4 receptors may help elucidate the exact nature of the EP receptor systems present in these preparations.

CHAPTER 3

Investigation of the inhibitory EP receptors present on human monocytes and their role in some functional responses

CHAPTER 3.1

General introduction to monocytes and their functions

Monocyte/Macrophage

The blood monocyte is generally thought to be an immature member of the reticulo-endothelial system (Florey & Gowans, 1962). Monocytes and their differentiated tissue forms, the macrophages, collectively comprise the mononuclear phagocyte system. Macrophages are essential for organ moulding, wound healing, phagocytosis of senescent cells in leaking vessels and host defence against microbes and invading pathogens (Nathan, 1987). Macrophages also recognise and ingest apoptotic neutrophils which aids in the resolution of inflammation (Haslett et al.,)

Monocytes are normally present in the circulation in small numbers and account for 3-8% of the total leukocytes (Wintrobe, 1951). Their origin is in the bone marrow (Volkman & Gowans, 1965a & b) and they have a short half-life in the circulation. Monocytes leave the blood randomly by diapedesis and migrate to tissues where they differentiate into macrophages (Spector & Willoughby, 1963).

Monocyte production is activated by macrophage-specific colony stimulating factor (CSF-1, also referred to as M-CSF) which stimulates hematopoietic stem cells to form colonies containing monocytes and macrophages (Stanley *et al.*, 1978; 1983).

Mature monocytes are the largest blood cells, with a diameter from 16 to 18 μm and range in concentration from 200 to 800 cells/μl whole blood. The transit time in marrow from first precursive monoblast to mature monocyte is about 6 days; in comparison to the neutrophil, there is no marrow reserve of monocytes.

Monocytes travel in the blood nearly 80% of the time, making them readily available for activation by cytokines released from sites of inflammation. Monocytes migrate much slower than neutrophils and arrive in appreciable numbers at inflammatory sites only after hours to days, rather than the minutes to hours for the neutrophil. During inflammation, the rate of monocytopoesis is increased because of increased levels of M-CSF and autocrine IL-1 and the newly recruited monocytes undergo rapid differentiation often terminating in the

formation of large epitheloid cells, and sometimes accompanied by the generation of multinucleated giant cells.

Mononuclear phagocytes are recognised in the bone marrow on a morphological basis. They undergo a series of differentiation steps to form monocytes which are released into the bloodstream. Monocytes do not undergo further division after entering the bloodstream. Upon leaving the bloodstream, monocytes distribute widely throughout the body, differentiating into many forms for which a variety of names are used, including the broadly applied term macrophage, see Table 3.1.1.

Diapedesis of selected monocytes is triggered by IL-1 and TNFα several days after release into the circulation. During diapedesis monocytes transiently down-regulate receptors linked to phagocytic, respiratory and secretory functions as they migrate towards chemotactic signals. Once across the vascular barrier, tissue monocytes regain their functional capacity within 20 to 30 minutes, and transform to macrophages in response to surface activation by IFNγ (Nathan *et al.*, 1983)

Collectively, tissue macrophages outnumber blood monocytes by over 100-fold and are very large 25-50 μm across, and it is estimated that the greatest number, more then half of the total, are found in the liver as Kupffer cells.

Monocyte differentiation

Macrophages are monocytes that have undergone transformation after leaving the circulation and are generated by the bipotential stem cell. The stem cell is driven to spawn monocytes preferentially by M-CSF, whereas bacterial LPS stimulates expression of the G-CSF gene in the stem cell. Co-ordination of monocyte and granulocyte production is achieved by feedback regulation involving the two opposing CSFs and by a balanced synergy between IL-1 and TNFα. As macrophages are the major source of both M-CSF and G-CSF, they are capable of regulating their own production.

TISSUE	GENERIC NAME
Blood	Monocyte
Spleen	Macrophage
Lymph Nodes	Macrophage
Lungs	Alveolar Macrophages
Liver	Kupffer Cells
Brain	Microglia
Bone	Osteoclast
Renal Glomeruli	Mesangial Cells
Peritoneum	Peritoneal Macrophages
Cervix	Hofbauer Cells

Table 3.1.1.- Distribution of monocyte/macrophage and the different nomenclature associated with their distribution

There are several features which typify the differentiation of mature mononuclear phagocytes. They become larger in size, develop an active endoplasmic reticulum and Golgi apparatus and have many secondary lysosomes in their cytoplasm, all of which are indicators of increased biosynthetic and intracellular degradative activity as well as the secretion of various soluble products.

Capabilities of macrophages

Activated macrophages enlarge and spread out further, become hypermotile and release an arsenal of noxious secretions, including an intense and sustained oxidative burst. The respiratory burst accompanying phagocytosis leads to the formation of reactive oxygen metabolites including superoxide anion (O_2^-) and hydrogen peroxide (H_2O_2).

When macrophages come in contact with foreign matter it is either engulfed or trapped and neutralised by an array of lysosomal substances. Macrophages, unlike neutrophils, are designed to survive combat and are capable of ingesting micro-organisms, red blood cells and other material. The list of secretory products released by activated macrophages is greater than 100 kinds of molecules (Johnston, 1988), some are shown in Table 3.1.2.

High affinity binding sites for chemotactic stimuli have now been identified on the surface of mononuclear phagocytes as members of the G-protein-linked seven transmembrane family of receptors. They include receptors for products of the activation of the complement system, notably C5a, certain bacterial products and cell-derived substances including LTB_4 and platelet factor 4. Triggering of these receptors sets in motion a series of cellular events leading to the assembly of contractile and cytoskeletal proteins to allow the orientation of cells towards the source of the chemotactic stimulus.

Reactive oxygen intermediates
O_2^-
H_2O_2
OH^-
NO

Lipid mediators
PGE_2
$PGF_{2\alpha}$
PGI_2
TXA_2
LTB_4
LTC_4
LTD_4
LTE_4
PAF

Chemotactic stimuli
C5a
LTB_4
PAF
IL-8
MIP-2

Enzymes
Lysozyme
Plasminogen activator
Collagenase
Elastase
ACE
PLA_2
Cytolytic proteinase

Cytokines and their inhibitors
IL-1α and β
IL-1ra
IL-6
TNFα
IL-10

Other proteins
Fibronectin
Apolipoprotein E
CSFs
Erythropoietin
Haptoglobin
INFα
INFβ
PDGF

Table 3.1.2 - Some secretory products of mononuclear phagocytes

These chemotactic agents also stimulate a number of other responses in phagocytic cells including the formation of eicosanoids and the release of oxidants and hydrolytic enzymes, all of which may facilitate the emigration of monocytes from the bloodstream into sites of inflammation.

Once they have arrived at sites of inflammation mononuclear phagocytes remove inflammatory stimuli and mediate subsequent tissue repair by a number of highly integrated systems intended for the recognition and removal of inflammatory stimuli and infectious agents, see Diagram 3.1.1.

Human implications of malfunctioning

The intensity of microbicidal response to infection is stimulated by IFNγ and subdued by PGE_2, secretory products of T-cells and of macrophages themselves, that respectively enhance and suppress activation. Some organisms can resist the deadly potential of the secretory response by selectively stimulating PGE_2 secretion by both cell types, thereby quenching both oxygen-dependent and independent antimicrobicidal mechanisms. Included among pathways that are constitutively capable of surviving and even of replicating within macrophages are such diverse intracellular organisms as *Mycobacterium avium intracellulare*, *Histoplasma capsulatum*, various protozoa, and the human immunodeficiency virus (HIV) (Murray, 1988). Lymphocytes from patients with some of these infections are immuno-compromised and unable to secrete enough IFNγ to activate macrophage host cells, a defect that can be overcome by administration of either recombinant IFNγ or inhibitors of prostaglandin synthesis such as indomethacin. In the case of protozoa, macrophages actually provide sanctuaries essential to protozoan survival.

Monocyte/macrophages are also involved in atherogenesis, as the first sign of atherosclerosis in the vessel wall is the formation of a fatty streak, which consists mainly of lipid-loaded monocyte/macrophages, the foam cells (Gerrity, 1981; Schaffner *et al.*, 1980).

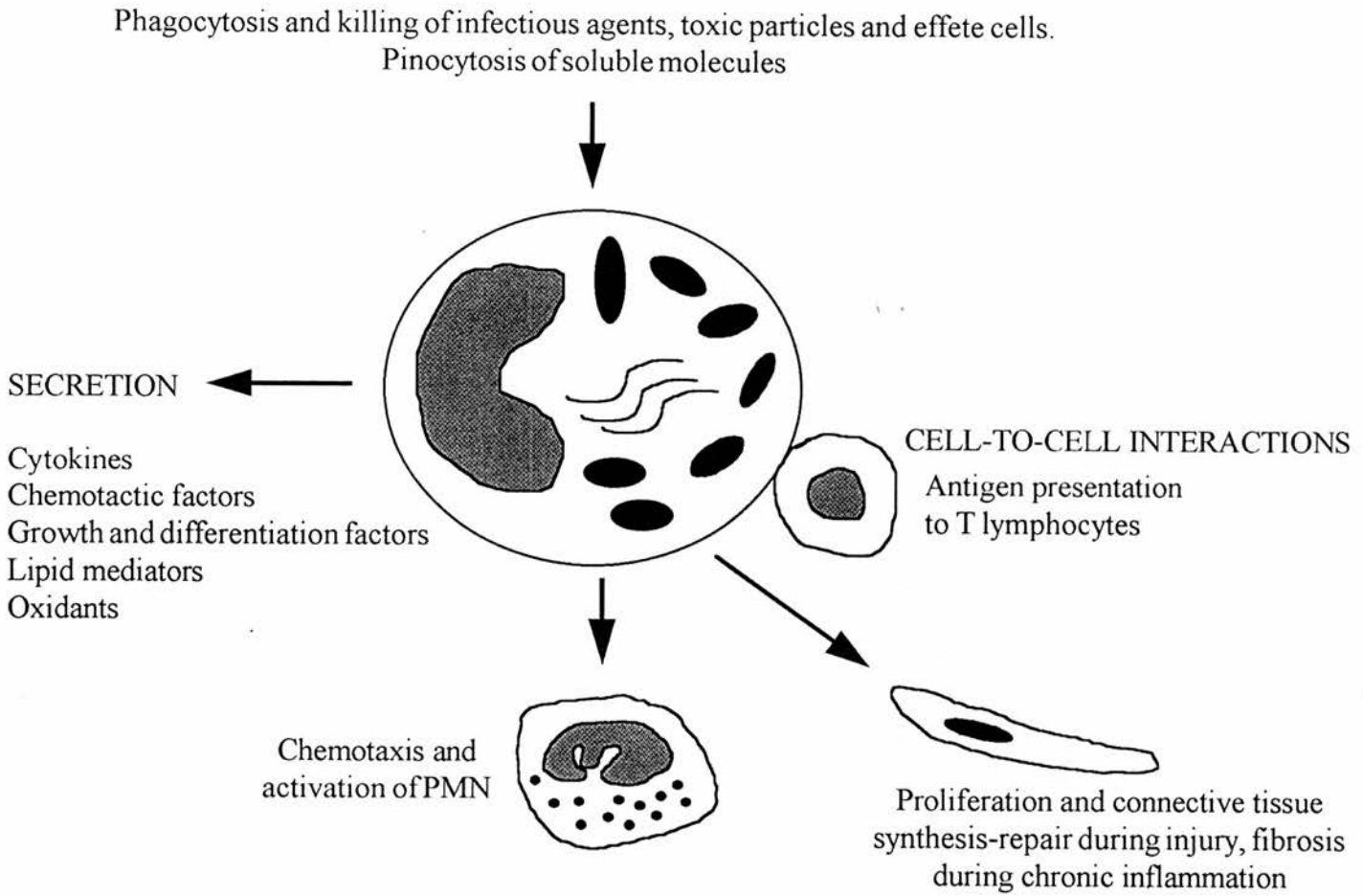

Diagram 3.1.1. - Some functions of the human monocyte/macrophage

Leslie & Dubey, 1994, investigated whether PGE_2 metabolism in the human monocyte was influenced by gender and by the stage of the menstrual cycle. Monocytes isolated from the female and activated *in vitro* with LPS produced on average significantly more PGE_2 into the medium than those from males. Among females, significantly more PGE_2 was found in the medium from cells isolated during the luteal phase of the cycle than during the early follicular phase. It was also in this luteal phase in which the female differed substantially from the males. They suggest that the *in vivo* hormonal changes associated with the menstrual cycle modulate monocyte synthesis of PGE_2 and other immune modulators such as IL-1. This could, they suggest, be a key to understanding differences in vulnerability between males and females to immune and inflammatory insult and also differences observed within phases of the menstrual cycle.

Prostaglandin production in monocyte/macrophages

Human monocytes are known to possess all the machinery required to synthesis cyclooxygenase- and lipoxygenase-derived eicosanoids (Schade *et al.*, 1989). PGE_2 was originally thought to be the major eicosanoid released by monocytes (Kurland & Bockman, 1978; Kennedy *et al.*, 1980; Bockman, 1981), but it was subsequently found that TxA_2 was the predominant eicosanoid metabolite, being produced in marked excess of PGE_2 (Pawlowski *et al.*, 1983; Orlandi *et al.*, 1989; Jones *et al.*, 1989). Allan and co-workers (Allan & Halushka, 1994) have characterised the binding site for TXA_2 on the human monocyte using [^{125}I]-BOP. Scatchard analysis reveals a single class of binding sites (K_d = 1.49 ± 0.14 nM and B_{max} = 696 ± 113 fmol/mg membrane protein). ^{125}I-BOP caused a dose-dependent increase in intracellular free calcium which was attenuated by preincubation with the prostanoid TP-receptor antagonist, SQ29548. The receptor which binds PGE_2 on human peripheral blood monocytes has also been characterised kinetically, although the subtype remains uncharacterised (Eriksen *et al.*, 1985). Kinetically, receptor binding was saturable, specific, and reversible at physiological temperature and pH and Scatchard analysis of binding data revealed a linear plot with a K_d of 1.1 pM and B_{max} of 4.1 fmol/10^7 cells. Exogenous PGE_2 addition increased intracellular cAMP by a maximal factor of three.

Whereas, $PGF_{2\alpha}$ and AA showed low binding to the cells and had no stimulatory effects on AC.

Another study showed that both human peripheral blood monocytes and murine peritoneal macrophages synthesise and release PGE_2 *in vitro*. This was markedly enhanced by LPS, and completely suppressed by indomethacin (Kurland & Bockman, 1978). In contrast, PGE_2 was not detected in the supernate fluids from cultures of human lymphocytes, granulocytes, T-, or B-lymphomas.

Aim

Treatment of monocyte/macrophages with PGE_2 induces increases in intracellular cAMP (Gemsa *et al.*, 1975; Bonta *et al.*, 1981). This increase in cAMP has been associated with inhibition of phagocytosis and shape change (Oropeza-Rendon *et al.*, 1980), inhibition of viral infection (Haraguchi *et al.*, 1995; van de Pouw Kraan *et al.*, 1995), oxidative burst (Elliott & Leonard, 1989) and osteoclast cell formation (Lacey *et al.*, 1995) in monocyte/macrophage populations.

Our aim was to classify the prostanoid EP receptor which mediates these inhibitory effects. The above results all implicate a role for cAMP in the inhibition of cell activation and this was used as a starting point. Initially, the cAMP assay system described in Chapter 2.1 was used to investigate the EP receptor positively coupled to AC present on human monocytes. After this we hoped to correlate this receptor activation with inhibitory functions of the human monocyte.

CHAPTER 3.2

Investigation of the EP-receptor mediating cAMP generation in the human monocyte

3.2.1.- Introduction

PGE_2 is released from the human monocyte and can act as a negative feedback control on many functions of the cells. It has been demonstrated that during infiltration of the macrophage into inflammatory tissue, the sensitivity of AC to activation by PGE_2 increases (Bonta *et al.*, 1981), and that in leukocytes, PGE_2 stimulates AC resulting in elevated intracellular cAMP levels and activation of PKA (Kammer, 1988).

Studying the effect of incubation on human blood monocytes, Coffey and co-workers, observed that PGE_2 stimulated increases of cAMP in freshly isolated monocytes and that this stimulation was attenuated in cells aged in culture 1-2 days (Coffey *et al.*, 1990). They observed heterologous desensitisation, since the AC responses to 5'-(N-ethylcarboxamido) adenosine, an A_2 receptor agonist, isoprenaline, a β-adrenoceptor agonist, and histamine also declined during culture. The observation that indomethacin prevented the desensitisation, suggested the role of a cyclooxygenase product. The above result indicate that, while PGE_2 inhibits monocyte functions via cAMP, its accumulation paradoxically permits cells to escape this regulation through a heterologous desensitisation of the cAMP response to itself and other agonists.

Monocytes/macrophages are associated with chronic inflammatory lesions, such as peridontal disease and rheumatoid arthritis, in which there is extensive connective tissue destruction. Matrix metalloproteinases (MMP) have been associated with the extensive tissue damage seen in diseases like rheumatoid arthritis. Mertz and co-workers observed that stimulation of human monocytes resulted in the production of MMP (Mertz *et al.*, 1994). The group found that IL-10, which modulates many functions of monocytes, decreased the production of MMP. IL-10 also decreased the production of PG by inhibiting COX-2 up-regulation, since the release of AA was unaffected by IL-10. Exogenously added PGE_2 or dibutyryl cAMP restored the production of MMPs in IL-10-treated monocytes. Additionally, COX-2 activity was restored by PGE_2 or dibutyryl cAMP, indicating that COX-2 is regulated through a PGE_2-cAMP amplification pathway. Another

study examined the effects of G-protein ADP-ribosylating agents, cholera toxin (CT) and pertussis-toxin (PT), on the signal transduction pathway that culminates in the production of monocyte MMPs (Corcoran *et al.*, 1994). They found that although CT elevated cAMP levels in both unstimulated and concanavalin A (Con A)-stimulated monocytes, it enhanced the production of COX-2 protein, PG, and interstitial collagenase only in Con A-stimulated monocytes. PT treatment suppressed the levels of cAMP, COX-2, PGE_2 and interstitial collagenase in Con A-stimulated monocytes. These two studies both suggested that activation of human monocytes induced production of MMPs is through a PGE_2-cAMP-dependent pathway.

Experimental allergic encephalomyelitis (EAE) is described as an autoimmune inflammatory disease of the CNS, and an animal model of MS. Misoprostol, and indomethacin, were tested and found to inhibit clinical and histological EAE (Reder *et al.*, 1994) and the combination of indomethacin plus misoprostol inhibited the disease further than each agent alone suggesting that cAMP elevating agents have an inhibitory role in EAE, and may also have a role in MS.

In HIV infection and allergic diseases, characterised by a dominant Th2 response, overproduction of PGE_2 has been observed. A recent study demonstrated that PGE_2 almost completely inhibited LPS induced IL-12 production, an essential chemical in induction of Th1 responses, whereas IL-6 production was only partially inhibited by PGE_2 (Van der Pouw Kraan *et al.*, 1995). In contrast, the production of IL-10 was enhanced approximately twofold under these conditions. The effects of PGE_2 in this system were due to its cAMP-inducing capacity, since they could be mimicked by other cAMP activating agents like dibutyryl cAMP and IBMX. This suggests that PGE_2 is a major inhibitory agent in HIV infection and that it mediated its effects via cAMP generation.

The effects of TNF-α on the regulation of M-CSF gene expression in human promyelocytic HL-60 cells were studied during monocytic differentiation with PGE_2 or dibutyryl cAMP (Sherman *et al.*, 1990). The combination of a protein synthesis inhibitor, cycloheximide, and TNF-α increased levels of M-CSF mRNA compared with treatment by

TNF-α alone. Inhibitors of PLA_2 activity blocked TNF-α-induced increases in M-CSF transcripts in a concentration-dependent manner, while inhibitors of the 5-lipoxygenase pathway, had no detectable effect on induction of M-CSF RNA. The group suggest that PGE_2 or dibutyryl cAMP treatment is regulated, at least in part, by both transcriptional and post-transcriptional mechanisms, and that PGE_2 and cAMP regulate transcriptional activation of the M-CSF gene by TNF-α. Interestingly, another study showed that theophylline concentration-dependently reduced TNF-α release by human monocytes and alveolar macrophages to the same extent (Spatafora *et al.*, 1994). Northern blot analysis demonstrated that theophylline was able to reduce TNF-α gene expression and the group proposed that, since TNF-α is known to be involved in the pathogenesis of bronchial hyperresponsiveness and asthma, the therapeutic activity of theophylline might be partly related to its effects on TNF-α release.

In monocyte/macrophages, increases in intracellular cAMP have generated different responses in the synthesis and release of IL-1α and IL-1β. IL-1α and IL-1β are peptide hormones produced by macrophages as well as several other cell types, which stimulate growth and differentiation of numerous cells including lymphocytes, neutrophils, fibroblasts, synovial cells, osteoclasts, hepatocytes and adipocytes (Oppenheim *et al.*, 1986). They are also potent fever inducers and have been suggested to play an important role in the pathophysiology of a variety of chronic inflammatory diseases (Dinarello, 1984).

cAMP was thought to inhibit IL-1 synthesis post-transcriptionally (Dinarello, 1989). However, more recent evidence has shown that cAMP analogues, and other agents that elevate cAMP, increase IL-1 mRNA accumulation and IL-1 production several-fold (Kassis *et al.*, 1989; Scales *et al.*, 1989; Sung & Walters, 1991). It has also been suggested that cAMP and cGMP play a role in the LPS-signal transduction pathway that triggers cytokine production and release (Molnar-Kimber *et al.*, 1993). However, when monocytes were stimulated by toxic shock supernatant, they synthesised less IL-1 when intracellular cAMP level were increased by PGE_2 (Knudsen *et al.*, 1986) and it seems that the effects of cAMP on IL-1 synthesis are stimulus dependent.

LPS is commonly used to stimulate IL-1 production in a variety of cell systems. Sung and Walters showed that peripheral monocytes and myelomonocytic cell lines could be stimulated by LPS to express IL-1 mRNA (Sung & Walters, 1991). They found dibutyryl cAMP, 8-bromo-cAMP, forskolin, cholera toxin, PGE_1, and PGE_2 synergised with LPS to increase the accumulation in cell lines of IL-1α and IL-1β mRNA by up to 50-fold. However, despite this marked increase in IL-1 mRNA accumulation, IL-1 protein synthesis in these cells was increased by only twofold suggesting that cAMP potentiates the expression of IL-1 mRNA but not its translation. Interestingly, increased intracellular cAMP has been demonstrated to have an inhibitory effect on the release of IL-1β after stimulation with LPS (Hurme, 1990; Viherluoto *et al.*, 1991; Verghese *et al.*, 1995b). Viherluoto and co-workers also observed only a small change in cellular IL-1β activity after increasing intracellular cAMP. Nevertheless, studies in astrocytes show a decrease in LPS stimulated IL-1β mRNA levels (Willis & Nisen, 1995) These results suggest that cAMP stimulates LPS induced IL-1 mRNA synthesis, whereas cAMP has little effect on the translation of IL-1 mRNA, and actually inhibits IL-1 release, in monocytes and monocytic cell types.

Direct PKC activation with phorbol-esters is also used to stimulate IL-1 release from monocytes and macrophage cell types. Increased intracellular cAMP in phorbol myristate acetate (PMA) stimulated cells augmented IL-1 mRNA expression markedly, and slightly increased release of IL-1 (Hurme, 1990; Sung & Walters, 1991) which suggests that the effects of increased cAMP are stimulus dependent.

Other cytokines are also affected by cAMP modulation. Inhibition of TNFα and IFNγ production have been demonstrated in human monocytes by PGE_2, whereas IL-6 transcription and release are increased in the same experiments (Bailly *et al.*, 1990; Haynes *et al.*, 1992).

Interestingly, PGE_2 in the presence of a phosphodiesterase inhibitor IBMX was capable of stimulating IL-1 and IL-6 gene expression and

protein production in monocytes (Serkkola *et al.*, 1992; Dendorfer *et al.*, 1994)

Receptor expression is also mediated by cAMP in monocyte/macrophages. Takii and co-workers (1992) showed that stimulation with human recombinant IL-1α or IL-1β increased IL-1 receptor mRNA. Exogenously added PGE_2 increased further the levels of both IL-1 receptor mRNA and intracellular cAMP indicating that PGE_2 also acts as a pro-inflammatory agent.

It has been reported that cAMP levels in inflammatory cells are regulated by a cyclic nucleotide PDE belonging to the PDE IV family, characterised by their selectivity for cAMP over cGMP and their sensitivity to the antidepressant drug rolipram (Livi *et al.*, 1990; Verghese *et al.*, 1995a). Verghese and co-workers also demonstrated that rolipram and Ro20-1724, selective PDE IV inhibitors, were the most potent compounds at enhancing cAMP levels and inhibiting the release of TNF-α and IL-1β in human monocytes (Verghese *et al.*, 1995b).

All the above data are conflicting as they show a major role for cAMP in both potentiating and inhibiting properties of the monocyte and macrophage. It is of important scientific interest to determine which EP-receptor subtype is linked to AC and thus mediates the effects of both endogenous and exogenous EP-receptor agonists. Additionally, previous studies have putatively characterised the AC coupled EP-receptor present on the human neutrophil as the EP_2 subtype (Wheeldon & Vardey, 1993; Talpain *et al.*, 1995),

We have attempted to characterise the EP-receptor subtype positively coupled to AC present on the human monocyte. A sensitive RIA was set up, as described in CHO cell methods, to directly measure intra- and extra-cellular cAMP from monocytes stimulated with an array of EP-receptor agonists. The effects of the EP_4-receptor antagonist, AH23848B, were also examined in this preparation.

3.2.2.- Methods

Monocyte extraction

1 - 200 ml of blood was removed from the forearm of healthy donors and heparinised with 100U/50 ml blood.
2 - Red blood cells were sedimented by mixing the blood 50:50 with 3% dextran and leaving to sediment for about 1 hr.
3 - The leukocyte-rich plasma was removed from above the red blood cell pellet and dispensed into 50 ml tubes. These were then centrifuged for 10 min at 220g to pellet the leukocytes.
4 - The supernatant was discarded and the pellet resuspended in 55% Percoll® before layering carefully onto a gradient of 81% (5 ml) and 70% Percoll® (3 ml).
5 - The gradients were centrifuged for 30 min at 450g to differentially separate the mononuclear cells from the neutrophils. The two layers were clearly visible, the mononuclear cells being the higher layer between the 70% and the 55% Percoll®. Each layer was pipetted into 50 ml tubes, diluted to 50 ml with 0.9% saline and centrifuged for 10 min at 220g.
6 - The mononuclear cells were resuspended in RPMI 1640 + 10% foetal calf serum (FCS) + 1% Penicillin/Streptomycin and seeded into 12 well plates (1.0 ml/well) and incubated for 1 hr at 37°C to allow the monocytes to adhere.
7 - The lymphocytes were removed by carefully removing the culture medium and gently washing the wells twice with sterile Hanks balanced salt solution (HBSS) before assaying for cAMP production.
8 - Drugs were added directly to the wells, in 1 ml aliquots of HAMS-F12 medium containing indomethacin 5 μM and IBMX 0.25 mM, immediately after washing and incubated at 37°C. If antagonist studies were being carried out, antagonists were added in 900 μl aliquots 10 min prior to the agonists, and drug dilutions added in 100 μl aliquots. Again all dilutions were made up in medium with indomethacin and IBMX, see Figure 3.2.1.

Radioimmunoassay

see Chapter 2.1

EC and EEC values

EC values were calculated relative to the maximum cAMP generation for PGE_2. EEC values were calculated by dividing the concentration of drug by the concentration of PGE_2 that generated the same increase in cAMP. All values were calculated separately for each experiment and pooled to give average ± SEM.

3.2.3.- Results

Initially a time course was carried out to determine the optimum time for incubation with PGE_2 (1 μM). Samples were taken from both the cellular and medium fractions to compare the data. The basal value for the cellular fraction was 40 ± 28 fmol cAMP/10^5 monocytes which increased to a maximum of 168 ± 36 fmol/10^5 cells after 5 min incubation before decreasing to 108 ± 32 fmol/10^5 cells after 30 min incubation, Figure 3.2.1. The medium fraction had a similar basal value of 43 ± 18 fmol cAMP/10^5 cells but only increased to a maximum of 78 ± 12 fmol/10^5 cells after 10 min. From these results 5 min was chosen as the incubation time and all samples were taken from the cellular fraction. All subsequent values are expressed as increases from basal.

Antagonist Studies

AH23848B is a weak antagonist at the EP_4 receptor and has been used in previous studies at a concentration of 30 μM (Chapters 2.1 & 2.2). In initial studies with the human monocyte AH23848B was tested at 30 μM with a 10 min preincubation time. AH23848B 30 μM inhibited PGE_2 induced cAMP production. This effect was significant (ANOVA, $p<0.05$), Figure 3.2.2a. Indeed the concentration-effect relationship for PGE_2 was considerably flattened, such that determination of CR and pA_2 were not possible.

AH23848B was also tested at 10 μM and while this apparently inhibited cAMP production in the presence of PGE_2, it was non-significant ($p>0.05$ with ANOVA). The PGE_2 curve shows concentration-dependent increases in cAMP to a maximum production of 1100 ± 850 fmol cAMP/10^5 monocytes with PGE_2 10 μM. This maximum production is lowered in the presence of AH23848B 10 μM to 780 ± 530 fmol cAMP/10^5 monocytes.

An apparent pA_2 was calculated from the results observed with AH23848B 10 μM since the shift to the right allowed calculation of EC_{50} values in the presence and absence of AH23848B. An apparent pA_2 of 5.58 was obtained, which compares favourably with those

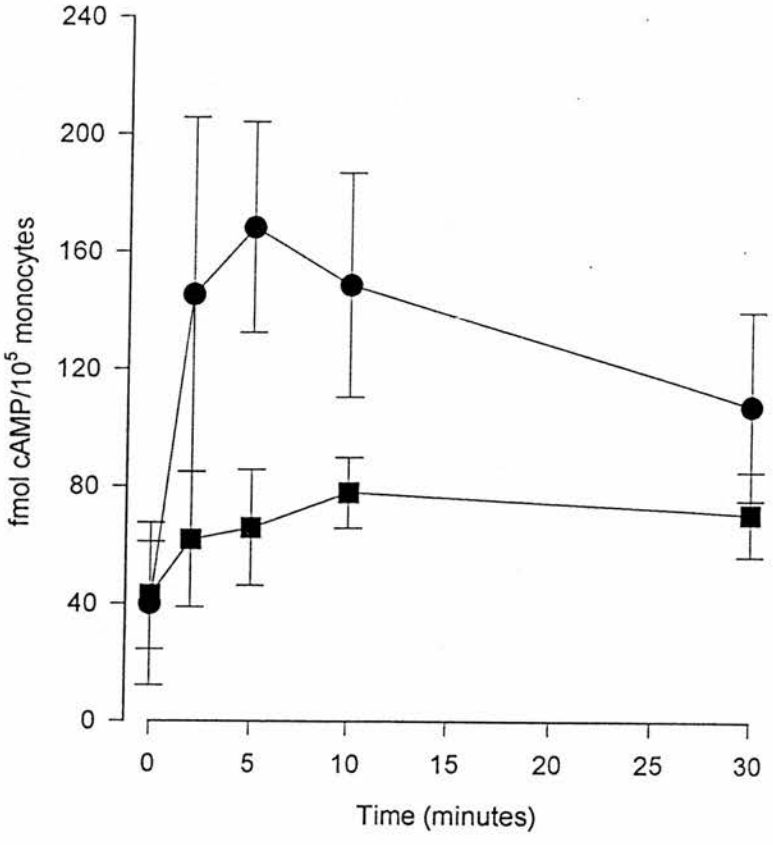

Figure 3.2.1.- Time course of PGE_2 (1 μM) stimulated cAMP production in human monocytes in cells (●) and medium (■), n=4. 5 min incubation generated a maximum increase in cAMP production in the cellular fraction of 168 ± 35 fmol cAMP/10^5 monocytes, this was chosen as the incubation time in all subsequent experiments and all samples were taken from the cellular fraction.

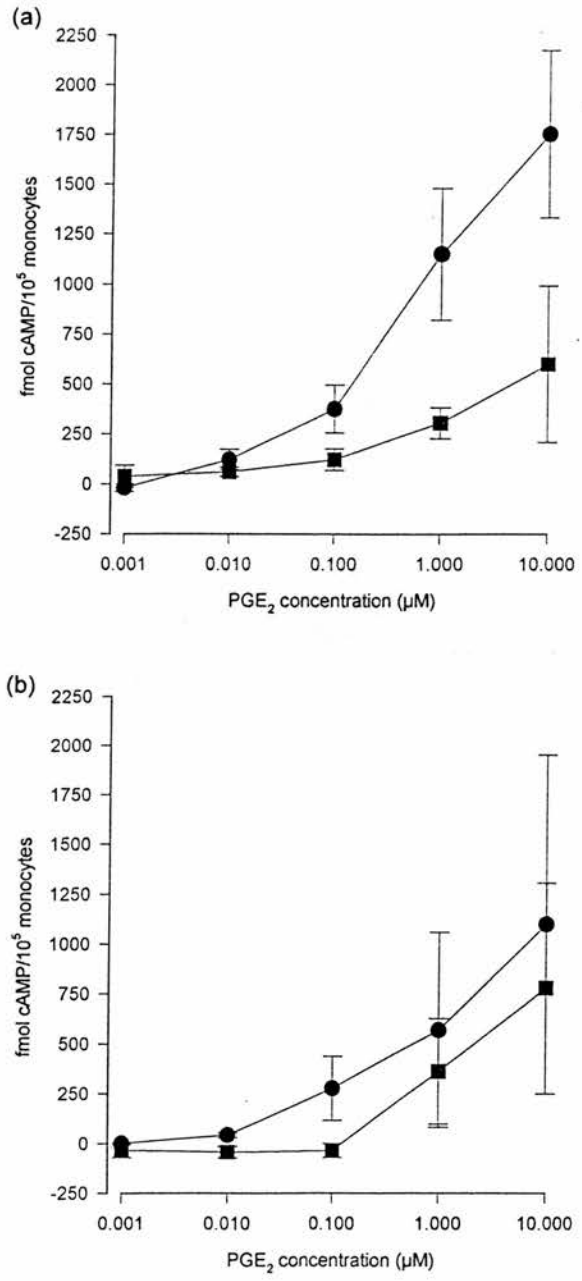

Figure 3.2.2.- PGE_2-induced increases in intracellular cAMP in human monocytes. (a) Effect of PGE_2 in the absence (●) and presence (■) of AH23848B (30 μM), n=3, ($p<0.05$ with ANOVA), and (b) Effect of PGE_2 in the absence (●) and presence (■) of AH23848B (10 μM), n=3, ($p>0.05$ with ANOVA, apparent $pA_2 = 5.58$).

obtained in other EP_4 receptor containing preparations, see Table 2.3.1, and suggests the EP_4 receptor subtype mediates PGE_2 induced cAMP generation in the human monocyte.

Butaprost 1 μM was examined for antagonism in this preparation and produced an apparent rightward, but non-significant, shift in the concentration-response curve for PGE_2 (ANOVA, $p>0.05$), Figure 3.2.3. Paired Student *t*-test point analysis of the maximum response observed with PGE_2 alone, and in the presence of butaprost 1 μM, however, gave a $p<0.05$ indicating that butaprost significantly inhibited the cAMP generation with PGE_2 10 μM.

Agonist studies

PGE_2, the standard agonist, concentration-dependently increased cAMP generation in human monocytes after 5 min incubation, Figure 3.2.4a 16,16-dimethyl PGE_2 and 11-deoxy PGE_1 also produced concentration-dependent increases in cAMP but were less effective giving lower EEC values than that of PGE_2, Figure 3.2.4a, Table 3.2.1. Butaprost and AH13205, in comparison, did not significantly increase cAMP in the human monocyte, Figure 3.2.4b. These results further indicate the involvement of the EP_4 receptor in cAMP generation in the human monocyte.

Misoprostol and sulprostone produced no significant increases from basal levels of cAMP, Figure 3.2.5a, Table 3.2.1, and it is unlikely that the cAMP generation in human monocytes is mediated by a AC coupled splice variant of the EP_3 receptor. PGD_2 and $PGF_{2\alpha}$ also generated no significant increases in cAMP levels, Figure 3.2.6.

Interestingly, nocloprost (up to 100nM) gave a concentration-dependent increase in cAMP equipotent with PGE_2, but with higher concentrations of nocloprost, no further increase in cAMP was observed. This suggests that nocloprost is a partial agonist in this preparation, Figure 3.2.5b, Table 3.2.1.

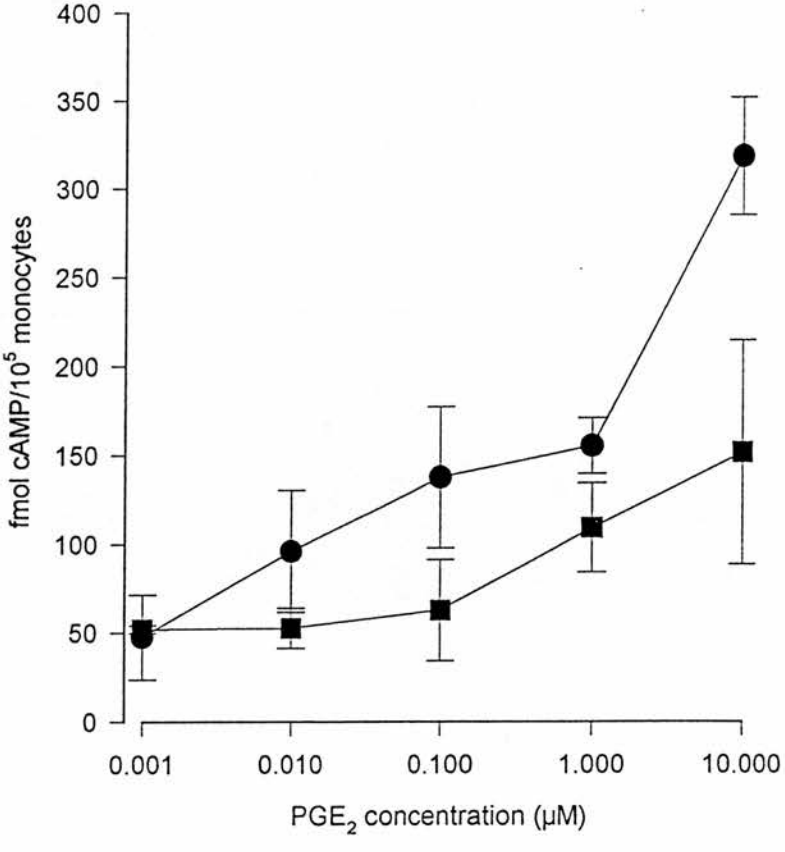

Figure 3.2.3.- PGE_2-induced increases in intracellular cAMP in human monocytes. Effect of PGE_2 in the absence (●) and presence (■) of butaprost (1 μM), n=3, ($p>0.05$ with ANOVA). Point analysis at PGE_2 10 μM showed a significant difference, $p<0.05$ with paired two-tailed Student *t*-test.

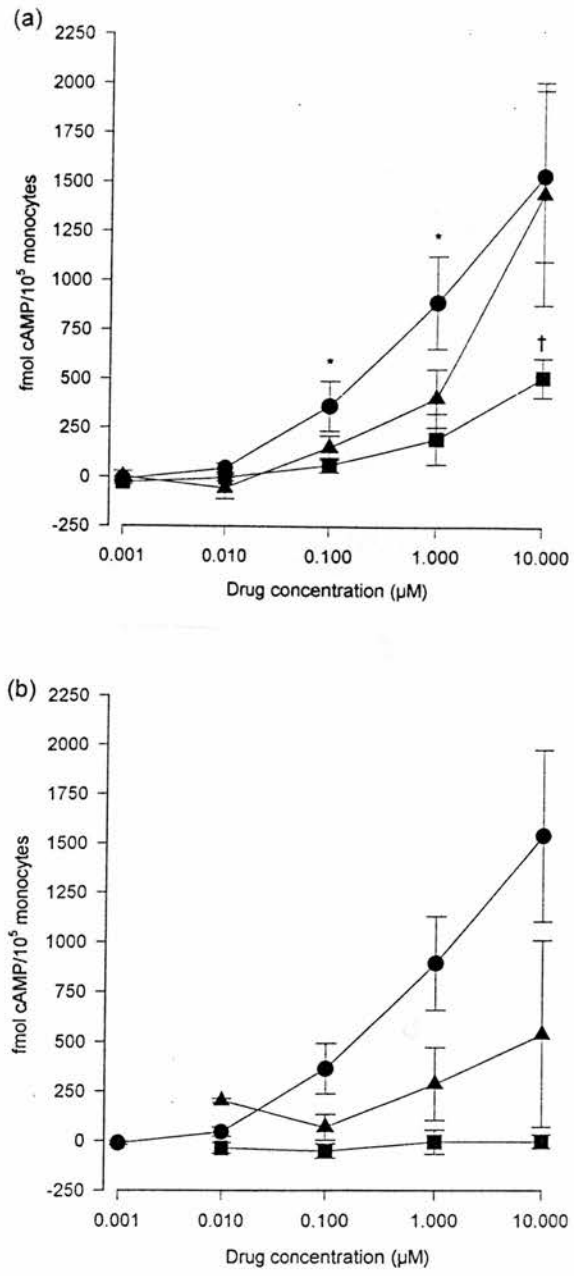

Figure 3.2.4.- (a) Effect of PGE_2 (●), n=8, 11-deoxy PGE_1 (■), n=5, and 16,16-dimethyl PGE_2 (▲), n=5, and (b) PGE_2 (●), n=8, AH13205 (■), n=6, and butaprost (▲), n=5, at increasing cellular cAMP in the human monocyte.

*- $p<0.05$ for PGE_2, paired one-tailed Student *t*-test.

†- $p<0.05$ for 11-deoxy PGE_1, paired two-tailed Student *t*-test.

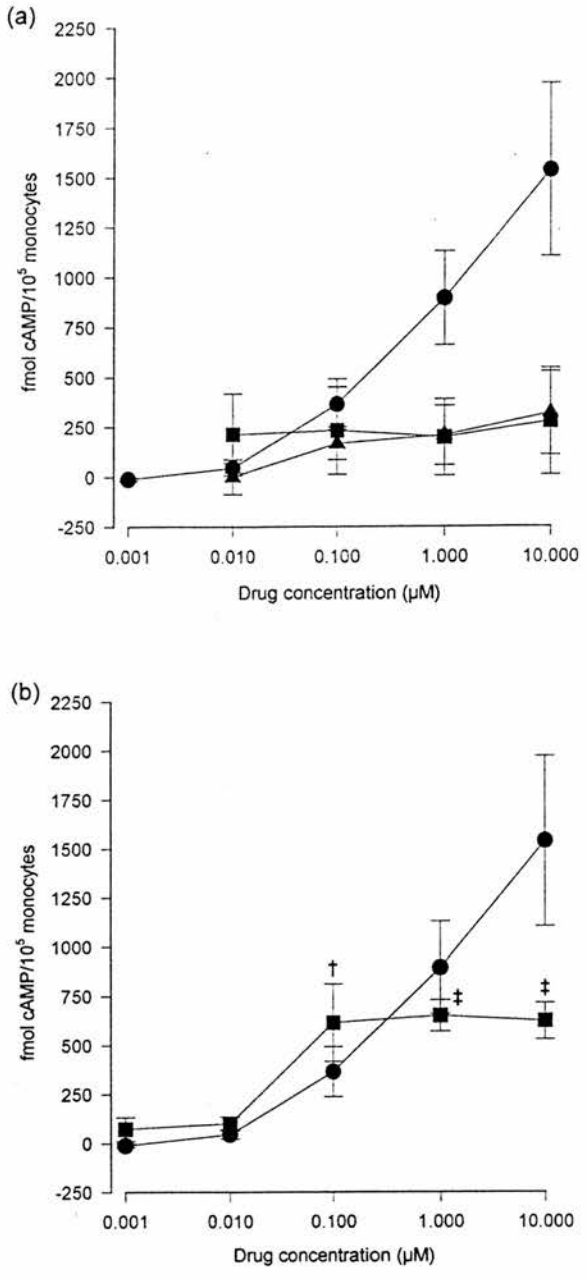

Figure 3.2.5.- (a) Effect of PGE_2 (●), n=8, sulprostone (■), n=4, and misoprostol (▲), n=4, and (b) PGE_2 (●), n=8, and nocloprost (■), n=4, at increasing cellular cAMP in the human monocyte.

†- $p<0.05$ for nocloprost, paired one-tailed Student *t*-test.

‡- $p<0.05$ for nocloprost, paired two-tailed Student *t*-test.

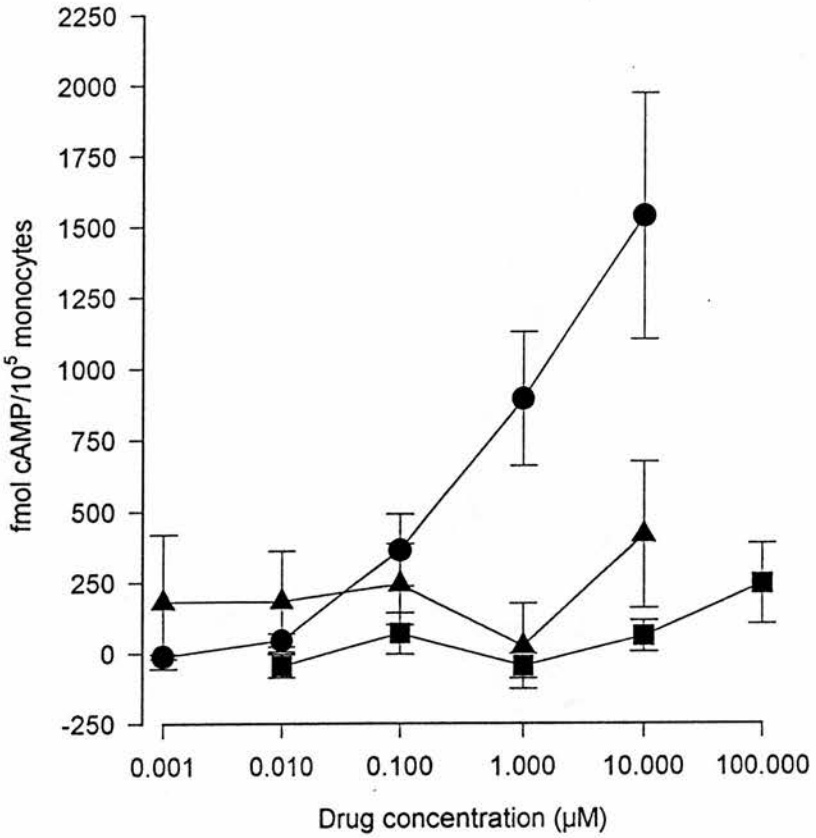

Figure 3.2.6.- Effect of PGE_2 (●), n=8, $PGF_{2\alpha}$ (■), n=4, and PGD_2 (▲), n=3, at increasing cellular cAMP in the human monocyte.

Agonist	EC_{30}	Maximum response (fmol cAMP/10^5 monocytes)	EEC (PGE_2 = 1.0)
PGE_2	390 ± 180 nM	1540 ± 430	1
nocloprost	40 ± 10 nM	650 ± 80	0.1
11-deoxy PGE_1	1.2 ± 0.5 μM	510 ± 100	3.1
16,16 dimethyl PGE_2	1.3 ± 0.6 μM	1450 ± 570	3.4
AH13205	>10 μM	10 ± 30	>25
butaprost	>10 μM	540 ± 470	>25
misoprostol	>10 μM	320 ± 210	>25
sulprostone	>10 μM	280 ± 270	>25

Table 3.2.1 - EC_{30}, maximum response and EEC values for a range of EP-receptor agonists at elevating cAMP in the human monocyte

3.2.4.- Discussion

Gemsa *et al.*, 1975, measured macrophage and medium cAMP from rat peritoneal macrophages. Their time course data for PGE_2 incubation showed a peak production in the cellular fraction after 5 min incubation (~30 ± 5 pmol cAMP/10^7 macrophages). This decreased after 30 min incubation and fell below the medium level after 60 min whereas the production of cAMP in the medium was a slow linear increase in cAMP. This compares favourably with our data for the human monocyte/macrophage.

AH23848B was used as an EP_4 antagonist to determine the effects of EP_4 receptor antagonism on cAMP production in human monocyte stimulated with PGE_2. AH23848B 30 μM produced a significant antagonistic effect. A lower concentration of AH23848B, 10 μM, was also examined and produced a small rightward shift in the PGE_2 concentration-response curve giving an apparent pA_2 of 5.58 which compares well with the pA_2 of 6.2 found in the CHO cells and with the pA_2s of 5.3 and 5.4 found in the PSV (Chapter 2.1 & 2.2; Louttit *et al.*, 1992a; Milne *et al.*, 1995; Coleman *et al.*, 1995). These data support the involvement of the EP_4 receptor in the generation of cAMP by PGE_2 in the human monocyte.

However, there is an alternative explanation which relates to the demonstarted TP agonist activity of AH23848B in some preparations (Brittain *et al.*, 1985; Lumley *et al.*, 1986). In this context, AH23848B may not necessarily be acting as an EP_4 antagonist in this preparation and may instead be acting as a physiological antagonist. TP receptor activation could inhibit any EP receptor stimulated increases in cAMP either by G_i activation so inhibiting AC activation, or by increasing $[Ca^{2+}]_i$ and indirectly inhibiting the cAMP activation pathway.

Butaprost was examined in the human monocyte for antagonist activity and inhibited the production of cAMP only at the highest concentration of PGE_2 used (10 μM). However, these data do not allow us to conclude which receptor is mediating this response.

The EP_2 receptor agonists butaprost and AH13205 did not significantly increase the levels of cyclic AMP in the human monocyte, Figure 3.2.4, Table 3.2.1. In EP_2 receptor containing preparations these compounds have EEC values of between 10 and 30 relative to PGE_2. Here, butaprost and AH13205 produced EEC values of >25, confirming that the cAMP response is mediated by EP_4 receptors in the human monocyte, Table 3.2.2. Misoprostol, another EP_2 agonist also had no effect in this system. Interestingly, misoprostol displaced bound [^{3}H]PGE_2 from COS-7 cells expressing the mouse EP_4 receptor (Honda *et al.*, 1993), which suggests that it binds the EP_4 receptor but has little or no efficacy.

11-deoxy PGE_1 concentration-dependently increased cAMP in this preparation which is consistent with its agonist activity at the EP_4 receptor and 16,16-dimethyl PGE_2, although non-significant, generated a maximal cAMP production similar to PGE_2. Neither compound, though, was equipotent with PGE_2, as was seen with the PSV and, as they also demonstrated a lower potency in the CHO cell line, may suggest a larger receptor reserve of EP_2 receptors in the PSV than the human monocyte, Table 3.2.2.

Exclusion of AC coupled EP_3 receptors, possibly splice variants, has been demonstrated with the inactivity of sulprostone and misoprostol in this preparation. However, this does not rule out the existence of inhibitory EP_3 receptors on the human monocyte.

Nocloprost produced a partial agonist-like curve in this preparation, generating an increase in cAMP production, but only to 50% of that observed with PGE_2 and, although nocloprost has not been tested for partial agonism in this system, the curve was reproducible. This may, however, be due to activity at EP_3 receptors present in the human monocyte negatively coupled to AC which would inhibit cAMP production.

Agonist	PSV	CHO cell line	Human monocyte	Cloned EP_4[1†]	Cloned EP_2[2†]	EP_2 receptor[3]
	EC/IC_{50} nM					
PGE_2	2.05	94	390	20	43	80
	EEC					
11-deoxy PGE_1	2.0	134	3.1	7.3	1.9	13
16,16 dimethyl PGE_2	2.8	163	3.4	7.3	2.4	9.4-20
butaprost	42	>10,000	>25	>1200	9.5	17
AH13205	3100-11,000	>10,000	>25	n.t.	n.t.	29

Table 3.2.2.- Comparison of EEC values obtained for EP-agonists in a range of EP receptor containing preparations

n.t.- not tested
†-K_i values
1- Nishigaki *et al.*, 1995
2- Regan *et al.*, 1994
3- Cat trachea (Dong *et al.*, 1986; Gardiner, 1986; Coleman *et al.*, 1988; Nials *et al.*, 1993)

To test whether there is expression of negatively coupled EP_3 receptors in the human monocyte, the cellular cAMP levels would first have to be increased with AC activating compounds such as isoprenaline, which positively couples to AC, or forskolin, which directly activates AC. Once elevated cAMP levels could be measured, incubation with sulprostone or GR63799X would demonstrate EP_3 receptor activity if they decreased the observed elevation in cAMP.

These studies show that PGE_2 and other EP_4 agonists positively couple to AC to increase the levels of cAMP in human monocytes and are consistent with the involvement of the EP_4 receptor subtype in mediating cAMP production in the human monocyte.

This work confirms earlier findings that PGE_2 stimulates cAMP production in human monocytes (Gemsa, 1975; Pelus & Strausser, 1977; Oropeza-Rendon *et al.*, 1979; Bonney *et al.*, 1980; Oropeza-Rendon *et al.*, 1980). These groups demonstrated a concentration-dependent stimulation of cAMP levels in the presence of PGE_2 but did not investigate which receptor subtype was involved. The work here strongly favours the involvement of the EP_4 receptor subtype.

One potential use for EP_4 receptor agonists could be to selectively down-regulate human monocyte functions without affecting other leukocytes in the circulation, and so mimic the body by using one of its own down-regulatory signals. Previous studies have suggested that the neutrophils and eosinophils express the EP_2 receptor positively coupled to AC and that this may mediate inhibition of functional responses (Kita *et al.*, 1991; Wheeldon & Vardey, 1993; Talpain *et al.*, 1995; Dr. E. Milne, personal communication). It has been shown that PGE_2 is released by monocytes in response to stimulation with IL-1 (Takii *et al.*, 1992), and observed that CNS microglia cells stimulated with IFNγ produce IL-1β and PGE_1 (Porrini *et al.*, 1994), suggesting that PGE_2 acts as a negative feedback system.

Further studies are required to evaluate the functional role of the EP_4 receptor in the human monocyte and the inhibition of IL-1α and IL-1β release by PGE_2 was investigated to try and determine a physiological role for the EP_4 receptor.

CHAPTER 3.3

Investigation of the EP-receptor mediating inhibition of IL-1α and IL-1β release from human monocytes

3.3.1.- Introduction

Interleukin-1 (IL-1) is a group of hormone-like polypeptides mainly produced by cells of the monocyte/macrophage lineage after infection, injury, or antigenic challenge (Oppenheim *et al.*, 1986; Beutler & Cerami, 1987; Dinarello, 1988) and it has been shown that LPS-stimulated blood monocytes produced significantly more IL-1 than peritoneal macrophages (Friedlander, 1994). IL-1 is also produced by several other cell types including lymphocytes, neutrophils, fibroblasts, synovial cells, osteoclasts, hepatocytes, and adipocytes (Shirakawa *et al.*, 1988).

The cloning of cDNA for mouse (Lomedico *et al.*, 1984), rabbit (Furutani *et al.*, 1985) and human (Auron *et al.*, 1984; March *et al.*, 1985; Tocci *et al.*, 1987) IL-1 molecules has shown that there are two distinct forms of IL-1, IL-1α and IL-1β. In human monocyte/macrophages the IL-1 genes are not constitutively expressed, but their expression can be readily induced with a wide variety of factors; bacterial LPS, muramyl peptides, silica and urate crystals, phorbol esters and other cytokines (Di Giovine & Duff, 1990).

Processing of IL-1 precursors appears to occur in conjunction with externalisation, since only 31 kDa precursors are found intracellularly, whereas the mature 17.5 kDa form is exclusively extracellular (Bayne *et al.*, 1986; Limjuco *et al.*, 1986; Giri *et al.*, 1985). The apparent absence of a leader sequence on the 31 kDa precursor, in addition to the observed accumulation of precursors for human IL-1 (Bayne *et al.*, 1986; Limjuco *et al.*, 1986; Matsushima *et al.*, 1986) and mouse IL-1 (Limjuco *et al.*, 1986; Gery *et al.*, 1981) within activated monocytes and macrophages, suggests that the mechanism of IL-1 secretion is distinct from that of typical secretory proteins. The IL-1 precursor in the cytoplasm needs to be processed prior to release, which may represent a novel pathway of protein secretion.

The IL-1α precursor is phosphorylated in LPS-stimulated murine macrophages and the principal, if not the only, site of phosphorylation is residue serine 90 (Beuscher *et al.*, 1988). The group also demonstrated that the 10% fraction of the phosphorylated IL-1α precursor that is membrane-bound is associated with lysosomal vesicles. Singer and co-workers found that IL-1β is not anchored to the plasma membrane, but localised in the cytosolic ground substance and showed that there were no significant amounts of IL-1β detected in the ER or Golgi apparatus (Singer *et al.*, 1988). Also, IL-1α, but not IL-1β, has been detected on the surface of activated human monocytes by flow cytometry using monoclonal antibodies specific to IL-1α and IL-1β (Conlon *et al.*, 1987). These data suggest that the intracellular precursors of IL-1, pre-IL-1α and pre-IL-1β are differently post- or co-translationally modified. Another group demonstrated this further by examining the phosphorylation of intracellular pre-IL-1 (Kobayashi *et al.*, 1988). It is known that the human IL-1α and IL-1β molecules are post-translationally cleaved from the 31 kDa precursor into 17.5 kDa biologically active molecules. They looked at LPS-induced production of both intracellular IL-1α and IL-1β in human monocytes. [^{32}P]-orthophosphate labelling of these cells revealed that pre-IL-1α was phosphorylated at least 10-fold more than intracellular pre-IL-1β. However, no [^{32}P]-incorporation could be detected in the 17.5 kDa processed IL-1α and IL-1β.

Analysis by thin layer chromatography revealed that the major phosphorylation site occurred at serine residue(s). The [^{32}P] was being incorporated into multiply cleaved precursors of IL-1α, which appeared in the absence of protease inhibitors and the smallest molecular weight pre-IL-1α that was labelled with [^{32}P] was 22 kDa, so the phosphorylated serine residue must be located adjacent to a sequence of four basic amino acids located in the 4 kDa region at the amino terminus of the 22 kDa precursor of IL-1α. This serine residue might also be a major phosphorylation site for a cAMP-dependent protein kinase. The group found that a synthetic peptide analogue of this region (residue 84 to 112) could be similarly phosphorylated *in vitro* by a cAMP-dependent protein kinase. In conclusion, Kobayashi

and co-workers suggested that there is no comparable amino acid sequence in IL-1β which could be expected to be phosphorylated by a cAMP-dependent protein kinase. Thus phosphorylation of truncated pre-IL-1α greatly enhances its susceptibility to digestion by trypsin, promoting the conversion of pre-IL-1α to the more biologically active IL-1α. Their findings suggest that the phosphorylation of serine close to dibasic/tetrabasic amino acid sequence functions to facilitate the processing and/or release of IL-1α.

It is now accepted that IL-1α and IL-1β are released through non-classical secretory pathways (Rubartelli *et al.*, 1990). Other studies have implicated protein kinase C and extracellular regulated kinase (ERK) signalling pathways as targets of LPS stimulation in different cell types (Weinstein *et al.*, 1992; Hurme *et al.*, 1990).

Cells that are stimulated by IL-1α and IL-1β include cells of the immune system as well as a wide range of non-immune cells. Responses of non-immune cells to IL-1 include proliferation of mesangial cells, keratinocytes, and fibroblasts, increased proteinase synthesis by chondrocytes, and increased synthesis of eicosanoids by fibroblasts, neutrophils, and monocytes (Dinarello, 1988, 1989; Mizel *et al.*, 1981; Canalis, 1986; Pasternak *et al.*, 1987; Dayer *et al.*, 1986; Newton & Covington, 1987). Immune cell-related actions of IL-1 include participation in the activation and functions of T cells, B cells, and NK cells (Gery *et al.*, 1972; Lipsky *et al.*, 1983; Herman *et al.*, 1985). IL-1 also enhances the response of T cells to other factors, particularly when these factors are at suboptimal concentrations (Gery *et al.*, 1972; Simon, 1984; Arya & Gallo, 1984; Matsushima & Oppenheim, 1985). IL-1β can even induce its own production through an autocrine loop (Schindler *et al.*, 1990).

When injected into animals IL-1β is an inflammatory cytokine causing fever, neutrophilia and other acute phase responses (Dinarello, 1988), and has proinflammatory and procoagulant activities (Dinarello, 1984). It is one of many cytokines that display immunologic, metabolic, hematopoietic, and inflammatory activities, both individually and in combination. IL-1β is a major effector

molecule in the pathogenesis of septic shock and bacterially-mediated local tissue destruction (Bone, 1991; Morrison & Ryan, 1987). IL-1β does not appear to be constitutively produced in healthy individuals, but is present in a number of disease states, including rheumatoid arthritis and infection (Dinarello, 1991).

The presence of IL-1β may have an important function in the development of rheumatoid arthritis by inducing the synthesis of other cytokines, proteolytic enzymes, and stimulation of fibroblast proliferation, leading to degeneration of the affected joint (Arend & Dayer, 1990; Shirakawa *et al.*, 1993). The synovial fluid from affected joints of rheumatoid patients has been shown to contain products primarily of macrophage origin. Some of the most prominent of these macrophage products are IL-1 itself, TNF-α, IL-6, M-CSF, GM-CSF and prostaglandins (Firestein *et al.*, 1990).

cDNA clones corresponding to the 80 kDa receptor for IL-1 were isolated by mammalian expression (Sims *et al.*, 1989). McMahan and co-workers reported the expression and isolation of human and murine cDNA encoding a second type of IL-1 receptor with a Mr of 60 kDa which they proposed to be a type II receptor for IL-1 (McMahan *et al.*, 1991). This mature type II IL-1 receptor consisted of (i) a ligand binding portion comprising three immunoglobulin-like domains; (ii) a single transmembrane region; and (iii) a short cytoplasmic domain of 29 amino acids. This last contrasts with the ~215 amino acid cytoplasmic domain of the type I receptor, and suggests that the two IL-1 receptors may interact with different signal transduction pathways. Another group has also shown that human monocytes express a 63 kDa IL-1 receptor type II that is immunologically distinct from the IL-1 receptor type I (Spriggs *et al.*, 1990).

It has been shown that treatment of human T cell/fibroblasts with IL-1α or IL-1β increased IL-1 receptor mRNA expression, PGE_2 release and a secondary accumulation of intracellular cAMP (Takii *et al.*, 1992). The exogenous addition of PGE_2 increased both IL-1 receptor mRNA and intracellular cAMP levels in untreated cells and forskolin, cholera toxin and 8-bromo-cAMP all increased IL-1 receptor mRNA

levels indicating that cAMP elevation can increase IL-1 receptor expression

IL-1ra was first reported in 1985 as an IL-1 inhibitory bioactivity of 22-25 kDa in the supernatants of human monocytes cultured on adherent IgG. IL-1ra is structurally related to IL-1α and IL-1β (26% & 30% nucleotide sequence identity, respectively) (Eisenberg *et al.*, 1991) and the cDNA has been cloned from a human monocyte library (Eisenberg *et al.*, 1990). Human IL-1ra has also been purified from supernatants of the human myelomonocytic cell line U937, after PMA stimulation (Carter *et al.*, 1990).

IL-1ra binds to IL-1 receptors, both type I and type II, on various target cells without inducing any specific biological response (Dripps *et al.*, 1991a & b). IL-1ra represents the first described naturally occurring cytokine that has specific receptor antagonist functions.

In contrast to IL-1α and IL-1β, IL-1ra has a classic secretory leader peptide and is glycosylated at a consensus N-linked glycosylation site (Hannum *et al.*, 1990; Poutsiaka *et al.*, 1991). The protein is therefore excreted through the Golgi-endoplasmic reticulum route. However, it has been described that up to 10%-50% of IL-1ra produced by monocytes remains cell associated at any time point during its synthesis (Granowitz *et al.*, 1992), and a truly intracellular form of IL-1ra without a signal peptide has been described in keratinocytes, but has not been detected in monocytes or monocytic cells.

IL-1α, IL-1β and IL-1ra bind with equal affinity to the 80 kDa IL-1 type I receptor present on T cells, endothelial cells and fibroblasts (Hannum *et al.*, 1990), and with 40-times lower affinity to the 67 kDa type II receptor found on human neutrophils, macrophages and B cells (Granowitz *et al.*, 1991). The specificity of IL-1ra, and the fact that it is produced by the same cells that produce IL-1, support the view that it is a physiologically significant regulator of IL-1.

Considerable information has accumulated pertaining to the blockade of IL-1 receptors using the naturally occurring IL-1ra (Dinarello,

1991). IL-1ra appears to be a pure receptor antagonist, since IL-1ra alone at concentrations as high as 1 μg/ml does not induce IL-1α, IL-1β or TNF-α synthesis in human monocytes (Granowitz *et al.*, 1992). In a Phase 1 trial in normal subjects, plasma IL-1ra concentrations of 25-30 μg/ml caused no symptoms or changes in vital signs and did not alter white blood cell counts or routine biochemical and endocrinological tests which is consistent with the concept that IL-1 does not play a role in normal homeostasis.

A particular protease has been described that appears highly specific for cutting the IL-1β precursor from 31 kDa to 17.5 kDa, its most active form (Dinarello, 1993). This enzyme is known as the IL-1β converting enzyme (ICE). ICE is a member of the cysteine protease family but it does not cleave the IL-1α precursor. Blocking ICE with ICE-specific substrate inhibitors reduced the amount of mature IL-1β produced by activated monocytes. So far, only peptide-based inhibitors of ICE have been identified, but there may be a therapeutic role for such agents as neutralising antibodies to IL-1β reduce endotoxin-induced fever in animals and decrease the proliferation of leukaemia cells *in vitro*.

One of the major outer membrane components of Gram-negative bacteria, LPS, is a potent activator of human monocytes and macrophages, resulting in the release of IL-1 (Krakauer, 1984; Nathan, 1987). LPS has been demonstrated to induce IL-1β mRNA transcription which is rapid, peaking within 2- 3 h and disappearing to barely detectable levels within 6 h (Fenton *et al.*, 1987). This rapid on/off regulation is thought to be characteristic of the regulation of IL-1β in monocytes.

LPS stimulation of monocytes has been found to be mediated through a cell surface protein, CD14 (Wright *et al.*, 1990). However, several studies have suggested that CD14 is not the only LPS receptor to be involved in the secretory response of LPS-stimulated monocytes (Raetz *et al.*, 1991; Couturier *et al.*, 1992). In support of this, Morrison and colleagues (1993) have shown the existence of more than one type of LPS-binding site on the monocyte membrane.

Studies have shown that LPS causes rapid tyrosine phosphorylation in human macrophages (Weinstein *et al.*, 1990) and that this is mediated by CD14 (Weinstein *et al.*, 1993). Mitogen-activated protein kinases and other proteins have also been identified as substrates for LPS-induced protein tyrosine kinase (PTK) activity (Dong *et al.*, 1993a,b; Weinstein *et al.*, 1992; Han *et al.*, 1993) and several studies have suggested that LPS activates pertussis toxin-sensitive G-proteins (Jakway & DeFranco, 1986; Zhang & Morrison, 1993). Protein kinase C (PKC) has also been found to play a role in induction of monocyte tumoricidal activity by LPS (Novotney *et al.*, 1991; Chung *et al.*, 1992; Taniguchi *et al.*, 1989; Coffey *et al.*, 1992; Dong *et al.*, 1989). Although, Coffey and co-workers (1992) have shown that LPS cannot stimulate phospholipase C (PLC) activity in human monocytes and these results suggest that LPS induces cellular activation via many second messenger pathways.

The signal transduction pathway involved in LPS-stimulated TNF-α and IL-1β secretion has been investigated in monocytes (Shapira *et al.*, 1994). TNF-α and IL-1β secretion were completely blocked by H-7, an inhibitor of both PKC and cyclic-nucleotide-dependent protein kinase, but were not affected by H-89, a specific cyclic nucleotide-dependent PKA inhibitor. LPS stimulation only slightly increased intracellular levels of diacylglycerol (DAG), the natural activator of PKC, and pre-treatment of monocytes with the DAG-kinase inhibitor, R59022, did not affect LPS-stimulated TNF-α secretion. LPS-induced PKC activation was also found not to be affected by blocking of CD14, the putative LPS receptor, with mAb or by inhibition of PTK with herbimycin A. However, these agents suppressed LPS-induced TNF-α secretion and TNF-α mRNA accumulation. The group suggest that LPS stimulated TNF-α and IL-1β secretion requires the activation of PTK and PKC, upstream to the activation of gene transcription in human monocytes. The activation of PKC by LPS, they suggest, is probably mediated by a DAG-independent pathway. Other groups have also shown that TNF-α and IL-1β secretion in LPS-stimulated human monocytes are dependent on both PTK (Dong *et al.*, 1993a & b; Geng *et al.*, 1993) and PKC activity (Novotney *et al.*, 1991; Chung *et al.*, 1992).

A number of endogenous agents are known to increase intracellular cAMP levels in the monocyte/macrophage (Hurme, 1990). Among these, the prostaglandins are possible compounds *in vivo* due to their high levels in inflamed rheumatoid arthritic joints (Seitz & Hunstein, 1985). It has been shown that exposure of human peripheral blood monocytes to the non-hydrolisable analogue of cAMP, dibutyryl cAMP, resulted in the induction of IL-1β mRNA production (Serkkola *et al.*, 1992).

Of the known intracellular second messengers, PKC activated by DAG is clearly involved in the activation of IL-1 gene expression: phorbol esters, which are structural analogues of DAG, bind to and activate PKC (Nishizuka, 1984), and are potent inducers of IL-1 production (Krakauer & Oppenheim, 1983; Strulovici *et al.*, 1989). In LPS- or IL-2-stimulated macrophages, IL-1 production can be down-regulated by pharmacological inhibitors of PKC (Kovacs *et al.*, 1988, 1989; Hurme & Serkkola, 1991). The role of cAMP as a second messenger in the induction of IL-1 expression has been extensively investigated and cAMP can also be an up-regulating signal: elevated cAMP levels induced either by prostaglandins or cell permeant structural cAMP analogues did not themselves activate the IL-1β gene but greatly potentiated the phorbol ester-induced IL-1β transcription and protein production both in human monocytes (Hurme, 1990) and in myeloid leukaemia cells (Hurme *et al.*, 1990).

Recent studies revealed that cAMP can also negatively regulate gene transcription for the human platelet activating factor, IL-2 receptor genes and LPS-induced transcription of tissue factor and TNF-α (Anastassiou *et al.*, 1992; Thivierge *et al.*, 1993; Bouvier *et al.*, 1989; Stockert, 1993; Taffet *et al.*, 1989; Ollivier *et al.*, 1993). IL-1β and TNF-α, once induced by LPS stimulation, activate secondary lipid mediators that elevate intracellular cAMP (Dinarello, 1989; Knudsen *et al.*, 1986) and it is possible that elevation of cAMP may mediate either a negative or a positive feedback mechanism on LPS-induced biosynthesis of specific cytokines in particular cell types (Knudsen *et al.*, 1986; Tannenbaum & Hamilton, 1989; Bailly *et al.*, 1990).

Peripheral monocytes and myelomonocytic cell lines stimulated by LPS or the phorbol ester PMA have been demonstrated to express IL-1 mRNA (Sung *et al.*, 1991). The group found that dibutyryl cAMP, 8-bromo-cAMP, forskolin, cholera toxin, PGE_1 and PGE_2 synergised with PMA or LPS to increase the accumulation in cell lines of IL-1α mRNA by up to 50-fold and that of IL-1β mRNA by 10- to 20-fold compared to LPS or PMA alone and suggested that the synergistic stimulation was due to enhanced IL-1 gene transcription rate rather than increased IL-1 mRNA stability. However, the same group found that the IL-1 protein synthesis in these cells was increased by only 2-fold by the cAMP elevating agents compared with the observed mRNA level and they suggest that IL-1 synthesis is under strict translational control.

The above experiments suggest that IL-1 mRNA production is positively regulated by cAMP. TNF-α is regulated translationally (Han *et al.* 1990) with the regulatory sequence residing in the 3'-untranslated region of TNF-α mRNA. IL-1 mRNA shares a number of sequence motifs with TNF-α mRNA in this 3'-untranslated region. It is possible therefore that similar mechanisms regulate IL-1 and TNF-α transcription and translation.

Human monocytes exposed to LPS generated a concentration- and time-dependent increase of IL-1 secretion in culture supernatants (Kassis *et al.*, 1989). PGE_1, PGE_2, cholera toxin and the PDE inhibitor IBMX, when added with LPS, resulted in a concentration-dependent increase in cellular cAMP and in secreted IL-1. Maximal levels of secreted IL-1 were 2.5-5.0-fold greater than that induced by LPS alone. These agents alone exhibited marginal effect on cell-associated IL-1. It was concluded by Kassis and co-workers that in adherent monocytes, IL-1 release is potentiated, and not inhibited, by prostaglandins or agents that elevate cellular cAMP, which differed from other groups results (Kunkel & Chensue, 1985; Brandwein, 1986; Knudsen *et al.*, 1986). They proposed the difference to be that they sampled from fresh adherent monocytes rather than mouse

resident macrophages (Kunkel & Chensue, 1985; Brandwein, 1986) or the U937 cell line (Knudsen *et al.*, 1986).

A more recent study showed in LPS-activated human monocytes that elevated cAMP concentrations, induced by PGE_2, forskolin or dibutyryl cAMP, reduced only secreted IL-1β, while the cell-associated level remained unchanged (Viherluoto *et al.*, 1991). cAMP also inhibited TNF-α production by monocytes, but the decrease was of the same magnitude in the extracellular and intracellular compartments indicating that the effect of cAMP on IL-1β release is due to the modification of the secretory process typical only for IL-1β.

It has also been demonstrated that the IL-1β production by LPS-activated human monocytes is readily inhibited by dibutyryl cAMP, without a significant change in the steady-state levels of IL-1β mRNA (Hurme, 1990,). By contrast, the same study showed that in PMA-activated monocytes dibutyryl cAMP increased IL-1β production ~4-fold, and the steady-state levels of IL-1β mRNA were also simultaneously increased. Interestingly, another study measuring the levels of TNF-α and IL-1 in response to LPS in human monocytes showed dibutyryl cAMP to have no effect on IL-1 production, but that dibutyryl cAMP did suppress concentration-dependently the production of TNF-α (Sato *et al.*, 1993).

Knudsen and co-workers showed that release of IL-1β activity by LPS can be completely blocked by dexamethasone in the monocyte-like tumour cell line, U937 (Knudsen *et al.*, 1987). Northern blot hybridisation analysis indicated that dexamethasone completely blocked accumulation of IL-1β-encoding mRNA. In addition, when cells were pre-stimulated with LPS, dexamethasone, did not change IL-1β mRNA levels, but completely blocked IL-1β release and induced a transient increase in cellular cAMP. This suggests that glucocorticoids suppress IL-1β synthesis by two distinct mechanisms, blocking transcription of IL-1β mRNA during monocyte activation and blocking post-transcriptional IL-1β via cAMP.

TNF-α is strongly implicated as an *in vivo* inducer of IL-1β. In monocytes, TNF-α appears to be an inducer of IL-1β production but only in the presence of dibutyryl cAMP or agents such as PGs which elevate intracellular cAMP (Lorenz *et al.*, 1995). The group found that this TNF-α/cAMP pathway regulates IL-1β production at the level of transcription, and requires a cAMP response element located between -2762 and -2755 bp in the upstream regulatory sequence of IL-1β. As PGs, which are known to increase cAMP levels *in vivo*, and TNF-α are both found in significant quantities in the synovial fluid of rheumatoid joints, this observed synergy may provide valuable insight into the potential pathways involved in the continuous production of IL-1β in the chronically inflamed joint.

PDE IV activity has been reported in monocytes and macrophages (Livi *et al.*, 1990). Verghese and co-workers have shown that the selective type IV cAMP-PDE inhibitor, rolipram, enhanced cAMP levels and inhibited the release of TNF-α and IL-1β in human monocytes (Verghese *et al.*, 1995). The non-selective PDE inhibitors IBMX and pentoxifylline were significantly less potent.

Another group studied the effect of selective PDE isozyme inhibitors on LPS-stimulated human monocyte viability and production of TNF-α and IL-1β (Molnar-Kimber *et al.*, 1993). The inhibitors used were vinpocetine (PDE-I), CI-930 and milrinone (PDE-III), rolipram and nitraquazone (PDE-IV) and zaprinast (PDE-V). None of the inhibitors affected monocyte viability at 10 μM or lower concentrations and only high concentrations of PDE-IV inhibitors modestly suppressed IL-1β production. PDE-IV inhibitors, and to a lesser extent PDE-III inhibitors, suppressed TNF-α production showing that selective PDE inhibitors can differentially affect the secretion of TNF-α and IL-1β from endotoxin-stimulated human monocytes.

The above results indicate that the control of IL-1 release is inducer-dependent, as well as second messenger-dependent. It is also likely that IL-1 production is affected differently by cAMP at its transcription, translation and release stages. The above data are also consistent with previous studies which show that TNF-α and IL-1β

are differentially regulated (Endres *et al.*, 1991; Tannenbaum & Hamilton, 1989). It is also feasible that PDE IV inhibition is a way of consistently inhibiting IL-1 release.

PGE_2 and PGI_2 analogues have been compared for their potency to influence LPS-stimulated production of IL-1β and TNF-α by human mononuclear cells (Eisenhut *et al.*, 1993). The stable analogues of PGI_2, iloprost and cicaprost, markedly suppressed TNF-α synthesis in LPS-stimulated mononuclear cells, without effect on IL-1β production. Although there was no significant difference in maximal suppression of TNF-α, iloprost and cicaprost suppressed synthesis to 50% of control at 20-fold lower concentrations than did PGE_2. The IC_{50} values for iloprost and cicaprost were 8 nM and 5 nM, respectively, compared to 125 nM for PGE_2 and elevated cAMP levels were observed with PGE_2 and cicaprost in this system. It is likely that human mononuclear cells also contain the IP receptor and that this is positively coupled to AC activation.

The ability to control IL-1 production either positively or negatively has great human implications. Decreased IL-1 production has been reported in patients with systemic lupus erythematosus (Whicher *et al.*, 1986), scleroderma, and malnutrition (Kauffman *et al.*, 1986), whereas i*n vitro* production of IL-1 appears to be increased in patients with rheumatoid arthritis (Wood *et al.*, 1985), idiopathic osteoporosis (Pacifici *et al.*, 1987), or major burns. It has also been observed that CNS microglia cells produce IL-1β, TNF-α, IL-6 and PGE_1, and that spontaneous and IFNγ-induced IL-1β secretion was greater in monocytes from patients with multiple sclerosis than in monocytes from normal patients (Porrini *et al.*, 1994).

Endothelial cells are also a target for IL-1, as IL-1 itself stimulates the proliferation of vascular smooth muscle cells. Hogquist and co-workers (1991) also demonstrated that while necrotic cell injury did not lead to the release of active IL-1β, apoptotic cell injury did.

Pleiotropic cytokines such as IL-1α have multiple effects on peripheral blood monocytes. Lee and co-workers examined the ability of *in vivo*

recombinant human IL-1α therapy to enhance clinically important monocyte functions in ovarian cancer patients prior to chemotherapy (Lee *et al.*, 1993). After 4 days of continuous infusion, *in vivo* IL-1α therapy amplified both the number and activity of monocytes. Therapy with IL-1α increased the number of monocytes six-fold, and these monocytes had a significantly increased ability to produce superoxide anion in response to phorbol 12,13-dibutyrate stimulation. Their ability to secrete spontaneously IL-1α and IL-1β was also significantly increased. The effects of IL-1α infusion demonstrated here should at least transiently increase the ability of monocytes to combat infection and enhance host immune response.

The aim of the present study was to find a receptor-mediated physiological event which was altered by PGE_2 and EP-receptor mimetics. This would allow the study of the EP-receptor function in a physiological system and also to study the second messenger system involved as well as investigate whether there is a correlation between increased intracellular cAMP and functional efficacy. If a working experimental system was obtained we then hoped to investigate further the EP_4-receptor present on human monocytes and to characterise the functional efficacy of EP-receptor mimetics studied previously. The effects of the EP_4-receptor antagonist AH23848B were also investigated.

IL-1 has been measured by RIA. Lonneman and co-workers (1988) set up a RIA for IL-1α, and Lisi and colleagues (1987) used one for IL-1β. Rordorf-Adam and co-workers (1989) have used an ELISA immunoassay to measure IL-1α and IL-1β from monocytes stimulated with LPS. Secreted IL-1β represented 31 to 86% of the total IL-1β and more IL-1α than IL-1β was produced but, unlike IL-1β, IL-1α was poorly secreted. IL release was measured here using ELISA kits selective for IL-1α and IL-1β.

LPS, the cytokine stimulus used in all experiments, was chosen because most current work on monocytes has been done using LPS. Other cytokine stimulants such as TNF-α, IL-6, A23197 (a Ca^{2+} ionophore) and opsonized zymosan have all been used in some experiments but the data are less complete and for initial studies, LPS was seen as a better starting point.

3.3.2.- Methods

Materials

Lipopolysaccharide (LPS) - LPS (*Escherishia coli* serotype 026:B6) was used in all experiments as the stimulatory agent at concentrations of 100 ng/ml (chosen after initial concentration-response curves, data not shown).
RPMI 1640 medium - Nutrient Mixture RPMI 1640, stored at 4°C.
Penicillin/Streptomycin (Pen/Strep) - Penicilin/Streptomycin solution, containing 10000 U/ml Penicillin and 10000 µg/ml Streptomycin, stored at -20°C.
Foetal Calf Serum (FCS) - Foetal Calf Serum Heat Inactivated. Mycoplasma and virus screened, stored at -20°C.
Cell Culture Medium - Cell culture medium was made up of RPMI 1640 medium (500 ml), FCS (50 ml) and Pen/Strep (5 ml), and stored at 4°C. The medium was always warmed to 37°C before use.
Culture plates - 12-well plates.
ELISA kits - IL-1α and IL-1β kits purchased from Cayman Chemicals, USA.
Drugs - All drugs were made up fresh each day in RPMI 1640 medium without FCS or Pen/Strep. These were warmed to 37°C in a water bath before use.
The antagonist AH23848B was made up at 1 mM and dissolved by sonicating for 15 min. After sonication the drug was immediately used.

Preparation of monocytes

Monocytes are very sensitive to trace amounts of LPS (Northoff *et al.*, 1987), so the possibility of LPS contamination in the reagents has to be considered. Human monocytes were purified from human blood as for cAMP studies, Chapter 3.2. Cells were again seeded to 12-well plates, and allowed to adhere overnight in cell culture medium. Monocytes were washed twice with 0.9% saline to remove any non-adherent contaminating cells.

Assay of monocytes for IL-1α and IL-1β

Studies involving cytokine production from human monocytes have used many different protocols. Some workers seed mononuclear cells to flasks, purify the monocytes by adhesion, and assay directly the pure monocytes in the flask. Other groups purify the monocytes by adhesion and scrape off the monocytes before assaying them in suspension at known concentration.

Initially, the monocytes were purified by adhesion and the flask scraped. The collected cells were centrifuged to give a monocyte pellet and, after counting, seeded at known concentration in 12 well plates. The cells were left to re-adhere before assaying. The advantage of having adhered monocytes was that they could be assayed for 24 hr in even contact with a drug without the need for a plate shaker. The cellular fraction could also easily be separated from the medium and by scraping the wells the concentration of adhered cells could be determined.

After initially adhering the cells however, it was apparent that there was a high concentration of dead cells after scraping (10-20% compared with <1% in the mononuclear cells before plating). It was decided to seed the mononuclear cell population directly to the 12 well plates at ~5 x 10^5 mononuclear cells per well. As the monocytes comprise 20-40% of the mononuclear cell fraction this usually left 1-2 x 10^5 monocytes per well.

The reaction was stopped by freezing. The medium was aliquoted from the wells to numbered tubes and the cellular fraction frozen dry. To resuspend the cellular fraction, HAMS-F12 (1 ml) was added before scraping with a rubber policeman.

Endres and co-workers (1988) found that lysing cells by repeated freeze-thawing yields maximal recovery of total (i.e., secreted plus cell-associated) immunoreactive IL-1β, when compared to extraction with the detergent CHAPS or addition of protease inhibitors. They

also observed that the soluble stimulus endotoxin induced twofold more IL-1α than IL-1β or TNF-α.

From these initial observations the decided protocol is outlined below. After 1 hr incubation, to allow the monocytes to adhere, all wells were gently washed with 0.9% saline. HAMS-F12 (1 ml) without FCS or Penicillin/Streptomycin was added containing LPS (100 ng/ml) and the cells incubated (2-12 hr). Drugs were added, in 1 ml aliquots of HAMS-F12, 5 min prior to addition of LPS (100 µl). In antagonist studies, antagonist was added 10 min before drug and LPS added 5 min after drug. All studies were carried out in the presence of 5 µM indomethacin.

The reaction was stopped on ice, and the cell supernatant removed to 4 ml plastic tubes and centrifuged at 320g for 10 min at 4°C to pellet any contaminating cells before storing at -20°C. The cellular fraction was removed by adding a further 1 ml of RPMI to each well and scraping with the rubber policeman. Samples were then removed to 4 ml plastic tubes, and frozen as for the supernatant. They were then defrosted on ice and assayed immediately for IL-1 using ELISA kits.

Initially, time course experiments were carried out to determine the optimum conditions and length of incubation with LPS. Time courses were stopped at 4, 8, 12 and 24 hr.

3.3.3.- Enzyme Linked Immunosorbant Assay (ELISA)

ELISA is based on a double-antibody 'sandwich' technique. Each well of the microtitre plates has been coated with either a monoclonal antibody specific for human IL-1α or 1β (capture antibody) depending on the kit. These antibodies will specifically bind any of their respective human IL-1 introduced into the well. An acetylcholinesterase: Fab' Conjugate (AChE:Fab'), which binds selectively to a different epitope on the IL-1 molecule is also added and 'sandwiches' the IL molecule between itself and the pre-coated monoclonal antibody. This effectively immobilises the bound IL on the plate and allows excess reagents to be washed away. The concentration of analyte can then be determined by measuring the enzymatic activity of the AChE with Ellman's Reagent which produces a yellow-coloured product absorbent at 412 nm.

Ellman's reagent consists of acetylthiocholine and 5,5'dithio-bis(2-nitrobenzoic acid). Hydrolysis of acetylthiocholine by acetyl-cholinesterase produces thiocholine and non-enzymatic reaction of thiocholine with 5,5'dithio-bis(2-nitrobenzoic acid) produces 5-thio-2-nitrobenzoic acid, which has a strong absorbency at 412 nm. The intensity of the colour is directly proportional to the amount of bound AChE:Fab' conjugate which is in turn proportional to the concentration of IL. Therefore by using a standard curve of human IL, the concentration of each sample can be accurately determined.

Acetylcholinesterase as the conjugate-linked enzyme has several advantages over other enzymes commonly used for enzyme immunoassays. Unlike horseradish peroxidase, acetylcholinesterase does not auto-inactivate during turnover. This property of acetylcholinesterase allows multiple development of the assay. In addition, the enzyme is highly stable under assay conditions, has a wide pH range (pH 5-10), and is not inhibited by common buffer salts and preservatives. The particular acetylcholinesterase used in this assay is from the electric organ of the electric eel *Electrophorus electricus* and is capable of high turnover (64,000 per second) for the hydrolysis of acetylcholine.

Materials

All water used for buffers and reagents was carbon filtered deionized water and all solutions were stored at 4°C unless otherwise specified.

EIA Buffer- The contents of one EIA buffer packet were dissolved in 500 ml of water.
Wash Buffer- The contents of one Wash buffer packet were dissolved in 500 ml of water and the packet rinsed twice. 0.25 ml of Tween 20 was added by a syringe.
Sample Matrix Blank (SMB)- This is the medium used for assaying the sample and standards. It is very important that this is IL free and matches the unknown samples to be assayed. As all my samples were in RPMI buffer, which is free of IL-1, I used this as my SMB and all the standard curve dilutions of IL-1 are made up in RPMI.
Interleukin Standards- The kit is supplied with 10 ng of IL-1. This is suspended in 2 ml of EIA buffer to give a stock solution of 5 ng/ml. An aliquot of 50 µl to 1 ml with SMB gives the first standard (S1) of 250 pg/ml the highest point on the standard curve. Sequential dilutions of S1 with SMB give 125, 62.5, 31.3, 15.6, 7.8 and 3.9 pg/ml making up standards S2-S7. The last standard S8 is only 500 µl of SMB and is the zero point of the standard curve.

Assay

1- 100 µl of standards and unknowns was added to each well in the plate followed by 100 µl of AChE:Fab' conjugate except the blank wells which were left dry.
2- The plate was then covered with plastic film and incubated overnight at 4°C.
3- To develop the plate the wells were emptied and rinsed five to six times with Wash buffer. Once dry Ellman's reagent (200 µl) was added to each well, including the blanks, and the plate covered with plastic film. The plates were always developed in the dark to limit the breakdown of Ellman's reagent.
4- Once the highest concentrations on the standard curve were visibly yellow (after 30 min) it was possible to obtain values for the

relatively concentrated samples by reading at 412 nm (405-420 nm). Longer development times, about 2 h, were necessary to obtain an accurate plot for the lower range of the standard curve and statistically significant values for sample concentrations near the detection limit of the assay (~1.5 pg/ml). The effect of the developing time is shown in Figure 3.3.1a.

5- After first subtracting the average blank value from each point, the result can be calculated by plotting a standard curve of absorbency versus concentration for the standards S1-S8 and constructing a best-fit line through the points, as shown in Figure 3.3.1b.

Specificity

The specificity of this assay is essentially 100% for IL-1α or IL-1β, depending on the assay kit used, and <0.01% for the other IL-1 subtype. Both assays have <0.01% specificity for IL-2.

3.3.4.- Results

Time Course Studies

2 hr incubation with LPS (100 ng/ml) produced more IL-1β in the cellular fraction than was released, Figure 3.3.2a. PGE_2 had no effect on released IL-1β but at 10 μM a slight inhibition in cellular IL-1β was observed. The amount of IL-1β released in the blank cells was the same as that for cells stimulated with LPS.

4 hr incubation with LPS (100 ng/ml) again produced high blank values in both fractions, Figure 3.3.2b. LPS produced 222 ± 47 pg IL-1β/10^5 monocytes , compared to 148 ± 50 pg IL-1β/10^5 monocytes in the blank cells and this LPS peak was significantly reduced by PGE_2 10 μM to 123 ± 31 pg IL-1β/10^5 monocytes.

Incubation for 8 hr with LPS (100 ng/ml) produced a similar profile to 4 hr incubation, Figure 3.3.3a. LPS significantly increased IL-1β production to 204 ± 54 pg IL-1β/10^5 monocytes from 100 ± 35 pg IL-1β/10^5 monocytes basal production and PGE_2 10 μM significantly decreased this LPS stimulated IL-1β production to 103 ± 27 pg IL-1β/10^5 monocytes.

12 hr incubation with LPS (100 ng/ml) produced only a slight increase in the cellular fraction IL-1β levels compared to the basal value and PGE_2 100 nM-10 μM had no effect, Figure 3.3.3b.

4 hr was chosen as the incubation time and was used for the rest of the experiments with both the cellular and medium fractions were sampled.

Agonist studies

4 hr incubation with LPS did not significantly increase the IL-1α production in the cellular or medium fractions compared to the basal value and PGE_2 (100 nM-10 μM) had no significant inhibitory effect on IL-1α levels in either fraction, Figure 3.3.4.

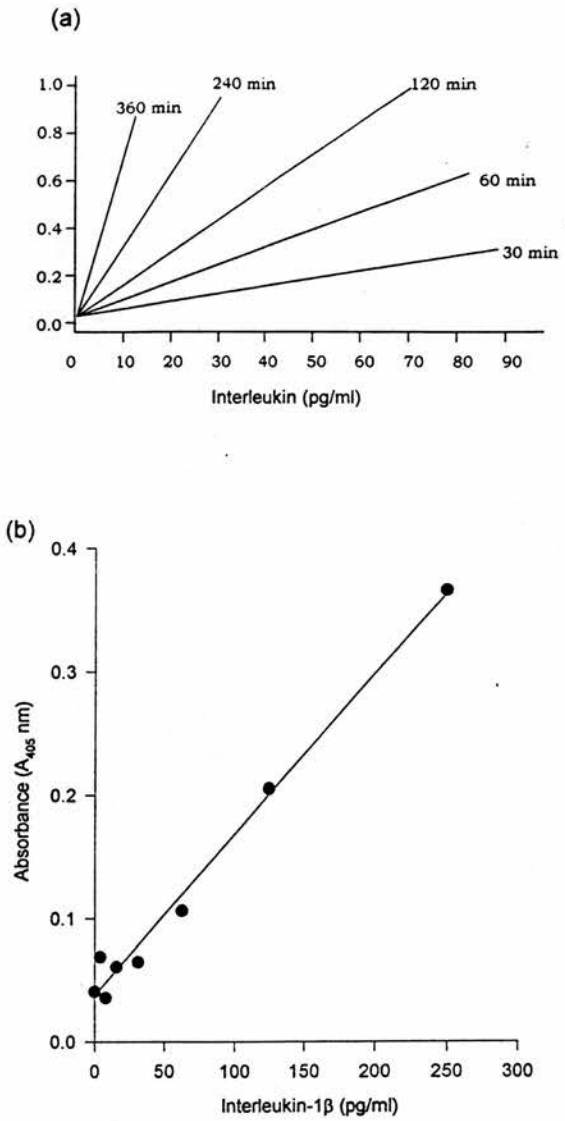

Figure 3.3.1.- (a) The effect of developing time on the standard curve. (b) Example standard curve for IL-1 production.

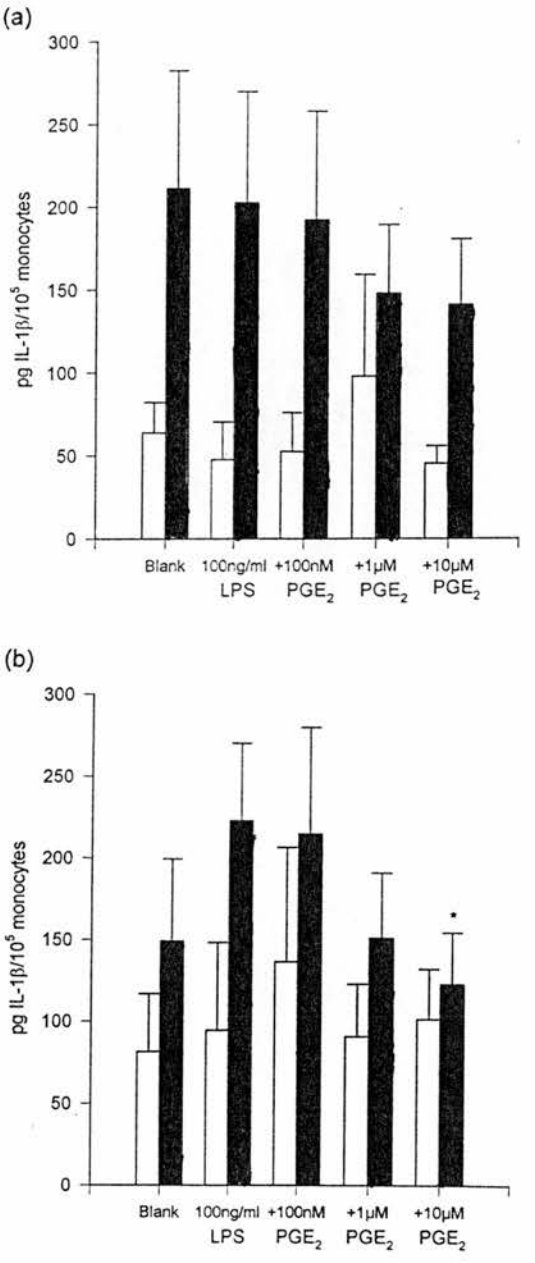

Figure 3.3.2.- (a) Effect of LPS 100 ng/ml (2 hours), n=4, and (b) LPS 100 ng/ml(4 hours), n=4, stimulation on cellular (shaded) and released (open) IL-1β production in the human monocyte.

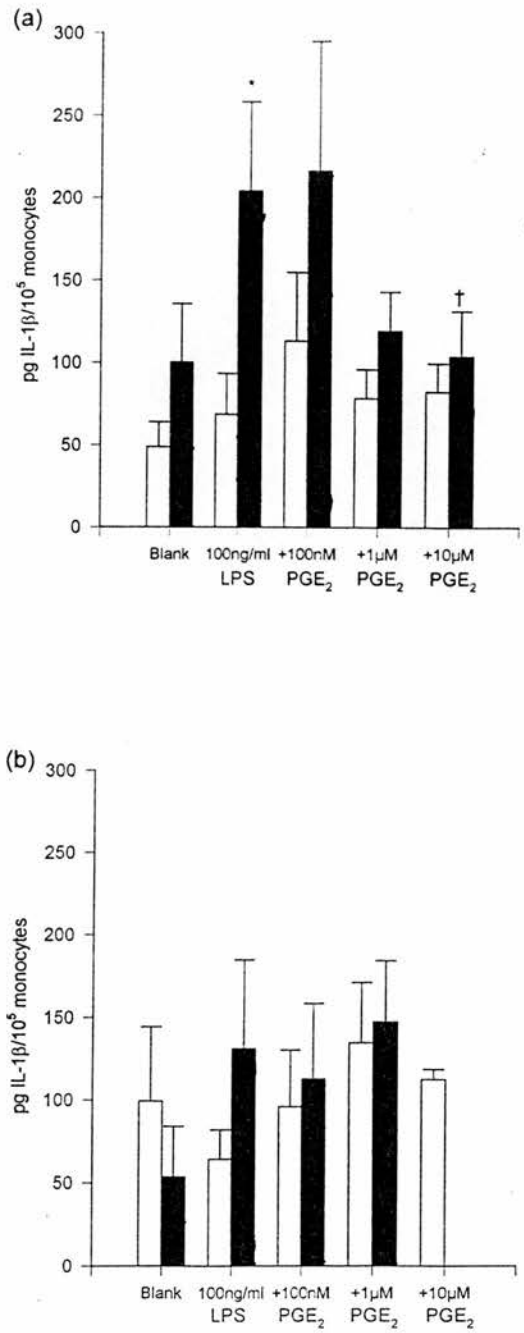

Figure 3.3.3.- (a) Effect of LPS 100 ng/ml (8 hours), n=4, and (b) LPS 100 ng/ml (12 hours), n=4, stimulation on cellular (shaded) and released (open) IL-1β production in the human monocyte.
*- $p<0.05$ compared to blank value, paired two-tailed Student *t*-test.
†- $p<0.05$ compared to LPS alone, paired two-tailed Student *t*-test.

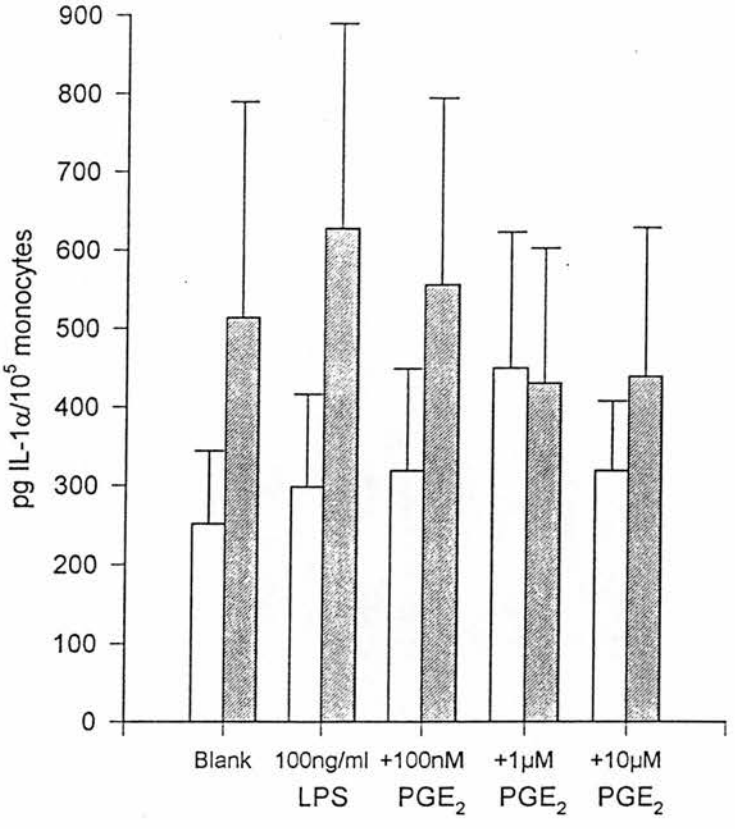

Figure 3.3.4.- Effect of LPS 100 ng/ml (4 hours), n=4, stimulation on cellular (shaded) and released (open) IL-1α production in the human monocyte.

Antagonist studies

Antagonist studies with AH23848B were performed to determine if the EP receptor mediating the inhibition of LPS induced IL-1 production was of the EP_4-subtype.

LPS, AH23848B and PGE_2 had no significant effect on the released levels of IL-1α, Figure 3.3.5a. However, in the cellular fraction PGE_2 alone produced no significant inhibition of the LPS induced IL-1α production but in the presence of AH23848B (1 μM) reduced the IL-1α levels to below basal production, Figure 3.3.5b. AH23848B alone had no effect on LPS induced IL-1α production, Figure 3.3.6.

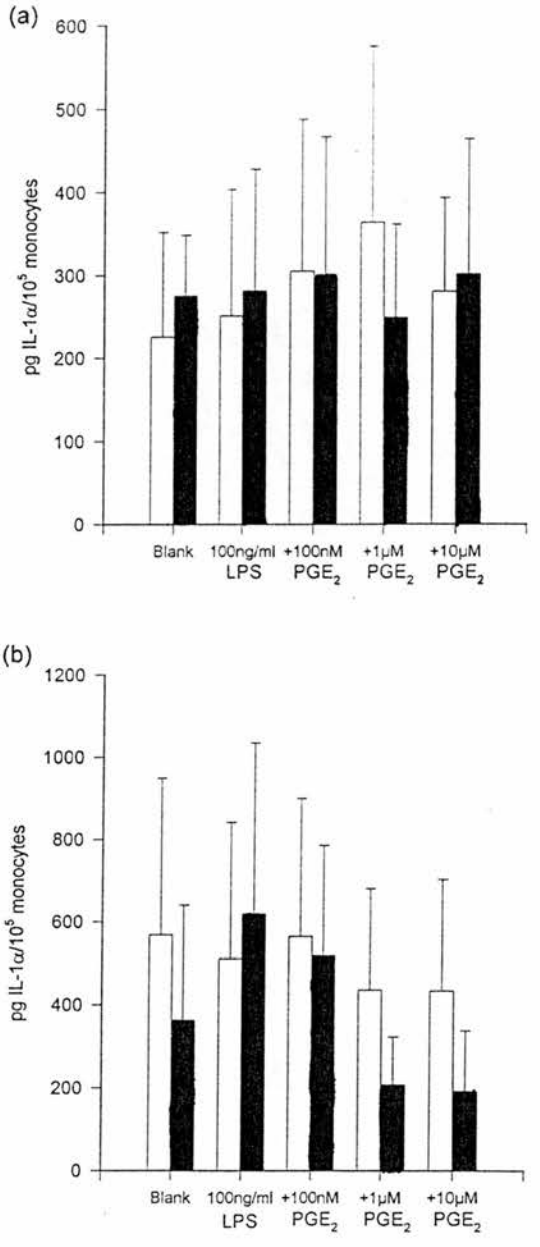

Figure 3.3.5.- (a) Effect of control (open) and AH23848B (1μM) (shaded) on released and (b) cellular IL-1α production in the human monocyte after stimulation with LPS 100 ng/ml (4 hours), n=3.

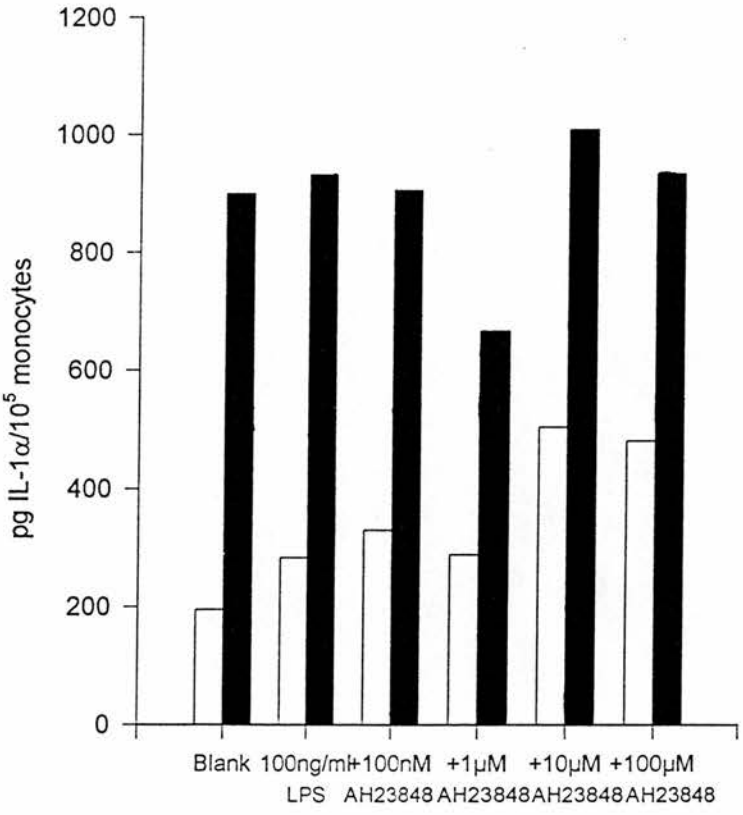

Figure 3.3.6.- Effect of AH23848B (100nM-100μM) on released (open) and cellular (shaded) IL-1α production in the human monocyte after stimulation with LPS 100 ng/ml (4 hours), n=2.

3.3.5.- Discussion

Experiments were carried out using this system to try an identify a functional inhibitory role for PGE_2 in the human monocyte and to provide a functional assay system to characterise the EP receptor involved. Our results demonstrated a significant production in IL-1β synthesis which was significantly inhibited by PGE_2, Figure 3.3.3.a. The results obtained in this system were, however, disappointing. In general the basal value was very high and LPS did not consistently stimulate the production of IL-1α or IL-1β.

The most intriguing finding in this study was that the cellular fraction generated higher levels of IL-1 activity compared to the medium, or released fraction. A possible explanation, for this relates to the nature of IL-1 activity. As preIL-1 is manufactured in the cytoplasm, and only converted to active IL-1 upon release, it could be possible that there are larger amounts of preIL-1, compared to active IL-1, in the cells which are converted to active IL-1 when the cells are lysed to assay for IL-1 production. This would explain the higher values observed in the cellular, over the released, fraction. Another possibility could be that the released active IL-1 remains bound to the cellular membrane and is thus detected in the cellular fraction rather than the medium. IL-1α activity has been detected on the surface of activated human monocytes by flow cytometry using specific monoclonal antibodies (Conlon *et al.*, 1987).

The blank values for IL-1 were very high in all the experiments. One reason for this could be that the monocytes were 'primed' before starting the experiments. It is likely then that they would be constitutively producing large amounts of IL-1 without an external stimulus. Lepe-Zuniga *et al.*, 1984, found that human monocytes, separated by adherence, and cultured in plastic wells, produced 'spontaneously' high levels of intracellular IL-1 during the first 20 hr in culture while releasing in most cases less than 10% of it into the medium. However, unlike our study, they found that the addition of LPS, quartz silica particles, opsonized zymosan, or PMA enhanced 3- to 50-times the overall production and 25- to 2000-times the release

of IL-1. They suggested that the reason for the different patterns of production and release observed with time might be that the synthesis and secretion of IL-1 by human monocytes are two distinct biological events, as mentioned in the Introduction 3.3.1. A possible reason for our small result was that Lepe-Zuniga and co-workers were using a thymocyte proliferation assay to measure IL-1 activity rather than an ELISA. With a thymocyte proliferation assay it is possible that factors other than IL-1 can stimulate proliferation. Bacterial infection of our media was ruled out, as fresh monocytes were used for each experiment and media with FCS was never stored.

Lepe-Zuniga and colleagues also measured a high level of IL-1 activity in the cellular fraction compared to the medium where as later studies indicate no IL-1 activity in cellular fractions (Giri *et al.*, 1985; Bayne *et al.*, 1986; Limjuco *et al.*, 1986). It seems likely that ICE activity is an important factor in these results as well as the use of ELISA to measure IL-1 activity compared with the older lymphocyte proliferation assays.

The functions of cAMP at regulating LPS induced IL-1 production have been hypothesised in Figure 3.3.7. This is a schematic drawing of the putative regulatory functions that cAMP exhibits against IL-1 synthesis and release in monocyte/macrophage populations. From this diagram it can be seen that, in general, increases in intracellular cAMP augment IL-1 mRNA synthesis by LPS but inhibit the translation and conversion/release of pre-IL-1 to its active 17.5 kDa form. Interestingly, prolonged elevation of intracellular cAMP can induce IL-1β synthesis in human monocytes (Serkkola *et al.*, 1992).

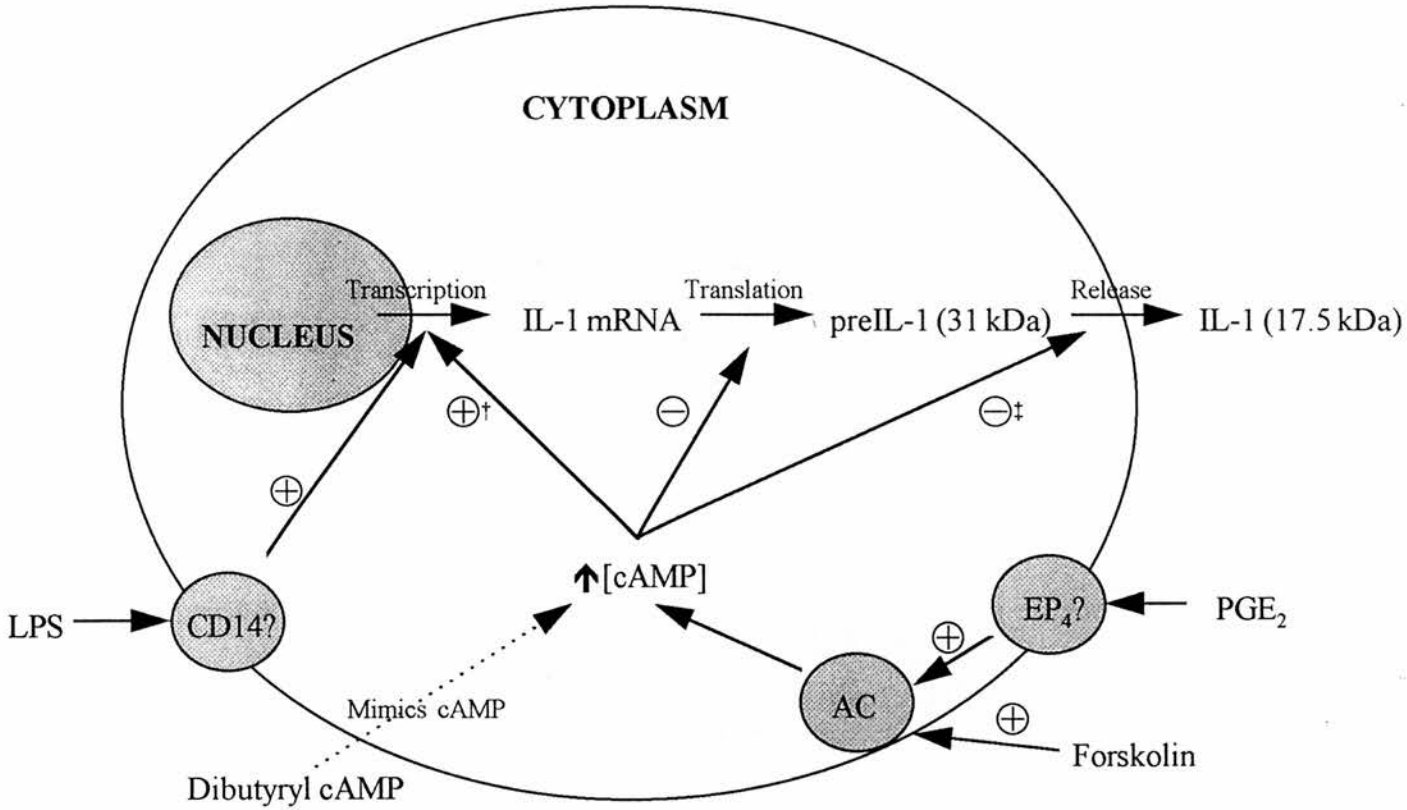

Figure 3.3.7 - Schematic illustration suggesting some of the effects of increased intracellular cAMP on IL-1 synthesis and release

† - has been demonstrated to synergise with other transcription activators
‡ - cAMP has been shown to potentiate IL-1 release, but this may be an artifact of the assay system used (Kassis *et al.*, 1989)

Post-transcriptionally, however, the two isoforms of IL-1 differ, as do the functions of cAMP on their LPS induced synthesis. In general, IL-1β release is inhibited by increases in cAMP (Viherluoto *et al.*, 1991; Molnar-Kimber *et al.*, 1993; Shapira *et al.*, 1994; Verghese *et al.*, 1995), but this may simply reflect the number of publications as most research has focused on IL-1β over IL-1α and earlier studies quoted generic IL-1 as assay systems did not differentiate between the two isoforms (Kunkel & Chensue, 1985; Brandwein, 1986; Knudsen *et al.*, 1986; Kassis *et al.*, 1989). Translationally, few studies have been carried, but it seems that IL-1α and IL-1β synthesis are under tight translational control as large increases in IL-1 mRNA do not produce similar increases in preIL-1, or released active IL-1 (Sung *et al.*, 1991).

This assay system did not seem suitable for our primary aim of receptor classification. Since IL-1 release is complex involving synthesis of a storage pool and release from that pool, it is difficult with this ELISA assay to discriminate between effects to inhibit synthesis or inhibit release. Additionally, a very small effect with LPS was observed due to a consistently high basal values. An assay measuring superoxide anion generation from human neutrophils was already being used in our laboratory (Talpain *et al.*, 1995), and it was decided to investigate this system in the human monocyte.

CHAPTER 3.4

Investigation of the EP-receptor mediating inhibition of superoxide anion release in human monocytes

3.4.1.- Introduction

Superoxide anion (O_2^-) generation is an important defence mechanism used by the monocyte. When monocytes are activated or ingest a foreign particle it is one of the reactive oxygen metabolites formed during the resulting respiratory burst and is generated in the monocyte by NADPH oxidase. NADPH oxidase is a membrane-bound flavoprotein which utilises NADPH as an electron donor and is helped by cytochrome b_{558}. The reaction catalysed is:

$$4O_2 + 2NADPH \rightarrow 4O_2^- + 2NADP^+ + 2H^+$$
$$2O_2^- + 2H^+ \rightarrow H_2O_2 + O_2$$

The NADPH is consumed to produce O_2- and by a glutathione-dependent H_2O_2 detoxifying system, is regenerated at the same time from glucose through the hexose monophosphate shunt. This reaction is characterised by the production of a large quantities of the oxygen radical O_2^- and hydrogen peroxide (H_2O_2) (Baehner & Johnston, 1971).

The products of the respiratory burst can react with endogenous products of oxidation to form, with halide cofactors such as bromide or chloride, highly toxic mediators, such as hydrobromous acid ($HBrO^-$), hydrochlorous acid ($HClO^-$) and other toxic products (Klebanoff *et al.*, 1980). These toxic reagents are able to kill parasites and bacteria (Klebanoff *et al.*, 1980), viruses (Belding *et al.*, 1970), mycoplasma (Jacobs *et al.*, 1972), and fungi (Lehrer, 1969). These metabolites can also destroy different kinds of human cells such as tumour cells and mast cells. They can also damage surrounding tissues (Spry & Tai, 1988) and act as proinflammatory agents (Baggiolini, 1984; Halliwell *et al.*, 1988). However, they have also been shown to inactivate mediators of inflammation such as leukotrienes (Henderson *et al.*, 1982).

Macrophages are 'primed' for enhanced oxygen radical release by macrophage activators like IFNγ and LPS (Pabst & Johnston, 1980; Nathan *et al.*, 1983), which do not themselves cause release of oxygen

radicals. Actual production of oxygen radicals by monocytes can be 'triggered' by phagocytosis, by contact with abnormal surfaces, or by exposure to chemical stimuli like FMLP, TNF-α, IL-1α, IL-1β and opsonized zymosan. Superoxide anion generation can also be stimulated in monocytes by direct PKC activation with PMA or PDBu.

The interaction between IFNγ and LPS on release of oxygen radicals by human monocytes cultured *in vitro* has been studied (Szefler *et al.*, 1989). After 48 hr culture, the group found that monocytes released low amounts of superoxide anion when stimulated by PMA or FMLP, however, monocytes incubated with either IFNγ or LPS became "primed" and released greater amounts of superoxide anion in response to PMA of FMLP. A similar observation was seen in a study measuring the induction of superoxide production and PKC activation in monocytes (Wolfson *et al.*, 1993). Wolfson and colleagues observed a strict correlation between superoxide production and PKC activation by PMA but found no correlation with IFNγ. However, pre-treatment of the monocytes with IFNγ for 10 to 15 hr enhanced both superoxide production and PKC activation with PMA. Using protein kinase inhibitors, they observed that while PMA exerted its effect by activating PKC, IFNγ operated via activation of a Ca^{2+}/calmodulin-dependent or some other Ca^{2+}-dependent protein kinases. Priming has also been demonstrated with GM-CSF, M-CSF, and IL-3 pre-treatment for 10 min which primed human monocytes and enhanced superoxide release stimulated by the receptor-mediated agonists, FMLP and concanavalin A, but not by PMA (Yuo *et al.*, 1992). However, Yuo and co-workers found that pre-treatment of monocytes with G-CSF, INFγ, or IL-4 had no effect on superoxide release stimulated by FMLP or concanavalin A. Thus, it would seem that monocyte priming may depend on the conditions of culture and length of exposure.

Human monocytes have been shown to contain the PDE IV isozyme with a K_m of 1.3 μM for cAMP and no cGMP hydrolytic activity (Thompson *et al.*, 1976; Elliott *et al.*, 1989). The involvement of cAMP in the reduction of superoxide anion generation is still unclear. It has been reported that sustained increases in intracellular cAMP induced

by PGE_1, IBMX, or dibutyryl cAMP inhibited FMLP-induced stimulation of superoxide (Smolen *et al.*, 1980). Interestingly, Elliott and co-workers measured the cellular content of cyclic AMP in monocytes treated with FMLP and adenosine, either singly or in combination (Elliott *et al.*, 1986). They found that FMLP induced a sustained increase in cellular cAMP compared with adenosine. The group suggested that the rise in cAMP produced by FMLP was via inhibition of PDE because the addition of FMLP to monocytes in the presence of adenosine caused a rise in cAMP that exceeded by a factor of two the sum of the increase seen with each agent alone. This, they argue, would not be expected if FMLP activated AC. It has also been shown in neutrophils that the FMLP induced rise in cAMP was not accompanied by an increase in the AC activity of neutrophil membranes (Verghese *et al.*, 1985). However, Elliott and co-workers showed that the increase in cAMP generated by FMLP was dramatically potentiated by the PDE inhibitor, rolipram, suggesting that PDE inhibition was not the mechanism by which FMLP induced a rise in cAMP (Elliott *et al.*, 1986).

Other groups have also investigated this phenomenon. One team showed that addition of the chemotactic peptide, FMLP, to a suspension of human monocytes caused a burst of superoxide release associated with a rise in intracellular cAMP, which occured within the first min of the burst (Leonard *et al.*, 1987). They also found that theophylline, a methylxanthine-derived PDE inhibitor, was a potent inhibitor of superoxide generation in monocytes. However, the rises in cAMP induced by theophylline or adenosine, which also inhibited superoxide generation, were small compared with that produced by the non-methylxanthine, PDE IV specific inhibitor, rolipram, which itself produced only a small inhibition of superoxide production.

In 1989, Elliott and co-workers looked more closely at the activity of the PDE IV isozyme and its relationship to the inhibition of superoxide anion generation (Elliott *et al.*, 1989). The action of oxidative burst inhibitors did not correlate with cAMP levels: rolipram, a PDE IV inhibitor, increased cAMP six-fold, with minimal

effects on superoxide generation, whereas theophylline increased cAMP less than two-fold but inhibited the oxidative burst by greater than 50%. Although theophylline and the AC activator, adenosine, inhibited FMLP-induced superoxide release, they did not inhibit production of inositol phosphates which suggests that their inhibitory activity is via the cAMP pathway.

To date, the cause of the increase in cell cAMP seen with FMLP has not been fully established. The relationship between cAMP production and inhibition of superoxide anion generation is still tenable, as all inhibitory compounds produce increases in intracellular cAMP. However, the exact relationship remains unclear, because the amount of intracellular cAMP generated, and the amount of inhibition observed do not correlate.

Pulmonary surfactant is known to have an inhibitory effect on the functions of monocytes. Geertsma and co-workers, examined its effect on human monocyte, and concluded that two protein kinases play a role in the regulation of the bactericidal functions of these cells. cAMP-dependent PKA is involved in inhibition, and Ca^{2+}/phospholipid-dependent PKC is involved in stimulation of these functions (Geertsma *et al.*, 1994). The significance of increased activation of PKA was demonstrated by the findings that surfactant induced a sustained rise in the intracellular cAMP concentration in monocytes, and that dibutyryl-cAMP mimicked the inhibitory effects of surfactant on both the killing capacity and the production of reactive oxygen intermediates by monocytes. Furthermore H-89, an inhibitor of PKA, partially restored the impaired bactericidal functions of monocytes incubated with surfactant. The same group also demonstrated the involvement of decreased activation of PKC in human monocytes treated with pulmonary surfactant. The group found that surfactant attenuated the PMA-mediated translocation of PKC, and that surfactant inhibited the production of superoxide by monocytes upon stimulation with PMA. Therefore, surfactant-induced inhibition of monocytes involves both activation of an inhibitory pathway, which includes cAMP and PKA, and inactivation of a stimulatory pathway, in which PKC is involved. PKA activation and

PKC inhibition, they suggest, may be mediated via a common pathway.

Li and Cathcart have studied the ability of human monocytes to oxidise native LDL and transform it to a cytotoxin (Li & Cathcart, 1994). They found that there was substantial protein phosphorylation induced upon monocyte activation. Pharmacological inhibition of PKC activity with the PKC inhibitors H-7, calphostin C, and GF109203X caused a concentration-dependent inhibition of cellular protein phosphorylation, including that of several previously identified PKC substrates. These inhibitors of PKC activity also substantially inhibited LDL lipid oxidation by activated monocytes which correlated very well with a profound suppression of superoxide anion production by these cells. Therefore it is possible that some of the potent substances which inhibit superoxide anion generation as described previously (Leonard *et al.*, 1987; Elliott *et al.*, 1986; Elliott *et al.*, 1989) may also act by inhibiting the activation of PKC.

The inhibitory effect observed on human monocytes by pulmonary surfactant (Geertsma *et al.*, 1994) is also present in other biological media. Skibinski and co-workers studied the effect of prostasomes, multilamellar vesicles produced by the acinar cells of the human prostate, on human polymorphonuclear cell and monocyte function (Skibinski *et al.*, 1992). They found that interaction of prostasomes with neutrophils and monocytes effectively inhibited superoxide anion generation in response to activation by PMA and FMLP. This interesting observation indicates that prostasomes, via inhibition of lymphocytes, may help prolong the life of sperm cells and enhance the chance of conception.

Monocyte viability *in vivo* is very important in the body, especially when there is exposure to external opportunistic infections. It is interesting to note how many biological systems exert inhibitory effects on mononuclear cells. A study found that VIP inhibited superoxide generation from FMLP-stimulated neutrophils, mononuclear cells and U937 cells in a concentration-dependent manner (Ishizuka *et al.*, 1992).

When working with monocytes *in vitro*, the effects of culture must also be considered. It has been described previously that monocytes cultured for 48 hr release low amounts of superoxide anion when stimulated with either PMA or FMLP and required stimulation with a 'priming' agent to elicit a greater response (Szefler *et al.*, 1989).

A group studying freshly isolated human monocytes found these were spontaneously cytotoxic towards K562 tumour cells (Martin & Edwards, 1993). They also found that superoxide radical production in response to either FMLP or PMA remained fairly constant for the first few days *in vitro* and then declined. NO_2^- concentration, however, in monocyte-conditioned medium was fairly constant during the first few days *in vitro* and increased after 6 days. They showed that this return to tumoricidal competence after 3 to 4 days in culture was decreased by the addition of L-NMMA. These results indicate that reactive oxygen intermediates are employed by monocytes in the killing of tumour cells and, after *in vitro* maturation of monocytes to macrophages, this mechanism becomes less important and reactive nitrogen intermediates are employed in mediating macrophage cytotoxicity.

Pabst and co-workers found that monocytes cultured in endotoxin-free medium M199 with or without 5% heat-inactivated autologous serum gradually lost their ability to produce superoxide anion in response to PMA over the course of 4 days (Pabst *et al.*, 1982), and that this diminished responsiveness to PMA was accompanied by decreased adherence and viability. The group found it was possible to prevent the loss of function, adherence, and viability by supplementing the culture medium with either bacterial LPS or muramyl dipeptide. They also found that the oxidative burst of monocytes cultured for several days without bacterial products could be partially restored by the addition of LPS on day 2 or 3 of culture.

As described above and by Bennett & Cohen, 1965, it is likely that the adherent monocytes used in these experiments are activated and have undergone some maturation to macrophages. They are likely

then to be 'primed' and will therefore elicit a larger superoxide anion generation in response to stimuli than quiescent monocytes in circulation.

As mentioned previously, phorbol esters and FMLP can stimulate superoxide anion generation in monocytes (Szefler *et al.*, 1989; Yuo *et al.*, 1992; Wolfson *et al.*, 1993). There has been no previous work looking specifically at the EP-receptor mediating inhibition of superoxide anion generation in monocytes, although it has been shown that PGE_1, PGE_2, PDE inhibitors and cAMP mimetics all inhibit FMLP-induced superoxide anion generation (Smolen *et al.*, 1980; Leonard *et al.*, 1987; Elliott *et al.*, 1989).

The aims of this chapter were, to measure the ability of PGE_2 and EP-receptor agonists to inhibit superoxide anion generation in human monocytes stimulated with phorbol esters or FMLP. We have also attempted to characterise which EP-receptor subtype(s) mediates inhibition of superoxide anion generation. Finally, the hypothesis that cAMP was the second messenger mediating the inhibitory effects of PGE_2 was also investigated.

3.4.2.- Methods

Monocyte extraction

Monocytes were obtained as described in the Chapter 3.2 methods, the only difference being that 24-well plates were used instead of 12-well plates

Measurement of superoxide anion generation

1- HBSS (900 µl) containing cytochrome C (2.5 mg/ml) containing indomethacin 5 µM was added directly to each well immediately after washing, and left to equilibrate for 10 min at 37°C.
2- Drug dilutions were all made up in HBSS with Ca^{2+} and Mg^{2+} and added in 100 µl aliquots to each well, and plates were incubated for 5 min. If antagonist studies were being carried out, these were added 10 min prior to the drug addition to allow the antagonist time to equilibrate. Blank wells with 100 µl of HBSS were always run in each assay, and in initial studies, superoxide dismutase (SOD) 90 U/ml blanks were also used.
3- Stimulant was then added to each well, and the plates left to incubate for 1 hr.
4- The reaction was stopped by aliquoting the medium to tubes on ice and leaving for 5 min. Tubes were then centrifuged for 10 min at 320g at 4°C.
5- After centrifugation, 200 µl aliquots of supernatants was dispensed into a 96-well plate, and the absorbance measured at 550 nm. The basal absorbance was taken as the average values from the blank wells. All experimental conditions were carried out in duplicate and all samples were assayed in duplicate. These were averaged to give a mean superoxide anion production from each experiment giving one n-value.

It has been reported that colchicine and other microtubule-disrupting agents stimulate IL-1α and IL-1β synthesis in human monocytes (Manie *et al.*, 1993). It is therefore likely that cytochalasin B, another microtubule disrupting agent used extensively in neutrophil

superoxide assays will do the same, and because of this cytochalasin B was not used in these experiments.

Statistical analysis

The Paired Student's *t*-test was used to measure the statistical difference between two points at a single drug dilution as this was analysing the effect of a drug on one variable. ANOVA was carried out on the concentration-response curve data for antagonist studies.

3.4.3.- Results

Superoxide anion generation can be stimulated by many compounds e.g. chemotactic peptides, zymosans and phorbol esters. Initially experiments were run using phorbol 12,13-dibutyrate.

Phorbol ester stimulated superoxide anion generation

Phorbol 12,13-dibutyrate (PDBu) stimulated superoxide generation of human monocytes in a concentration-dependent manner (EC_{50} = 33 ± 6 nM; maximum increase of 120 ± 10 nmol O_2^-/ 10^6 cells/ hr), Figure 3.4.1. From this curve it can be seen that 30 nM is a sub-maximal concentration and in further studies this was the concentration used.

Five min pre-incubation with PGE_2, the standard agonist, had no significant inhibitory effect on superoxide anion generation induced by PDBu, producing a maximum inhibition of about 9%, Figure 3.4.2a. Nocloprost, however, produced slight inhibition of PDBu activated superoxide release, 1 μM nocloprost caused a significant inhibition of 24 ± 7%, $p<0.05$ Paired Student *t*-test, Figure 3.4.2a. Butaprost, like PGE_2, had no significant effect on superoxide anion generation by human monocytes, Figure 3.4.2b.

FMLP stimulated superoxide anion generation

FMLP induced a concentration-dependent increase in superoxide anion generation (EC_{50} = 126 ± 110 nM; maximum increase of 90 ± 10 nmol $O_2^-/10^6$ cells/hr), Figure 3.4.1. From this curve 1 μM was selected as the stimulatory concentration and was used in all the subsequent studies. In comparison with PDBu, FMLP produced a similar concentration-response curve for superoxide anion generation. Interestingly, both stimulants at 10 μM produced a decreased maximal response, Figure 3.4.1.

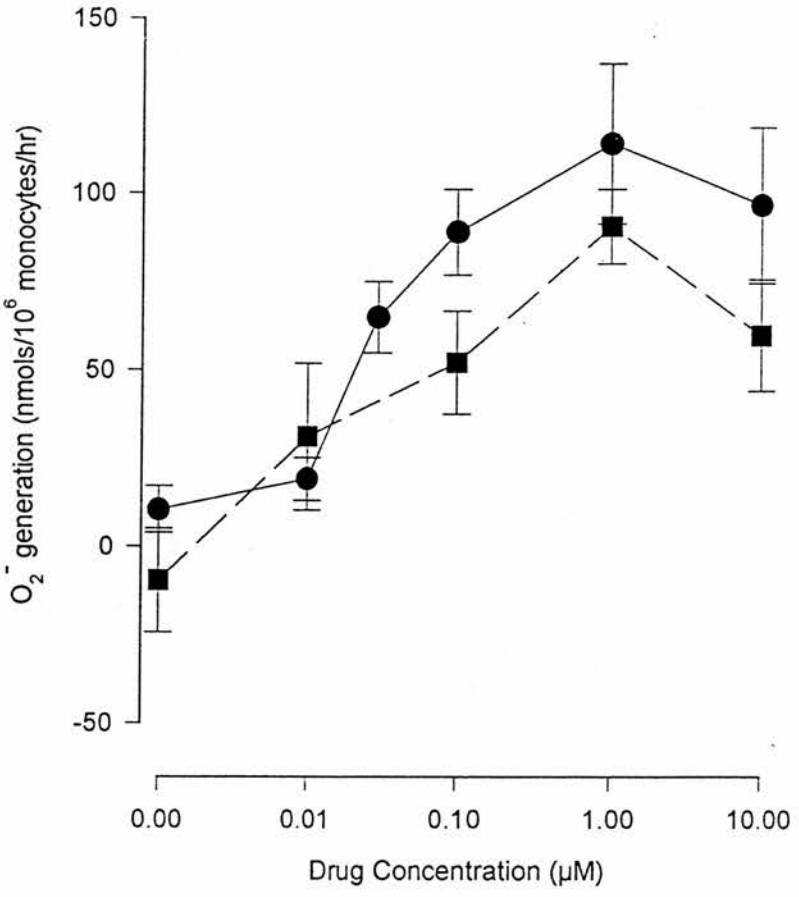

Figure 3.4.1.- Comparison of PDBu (●), n=7, and FMLP (■),n=4, stimulated superoxide anion generation in human monocytes. PDBu and FMLP generated maximum increases in superoxide anion generation of 121 ± 9 and 90 ± 10 nmol $O_2^-/10^6$ monocytes/hr respectively.

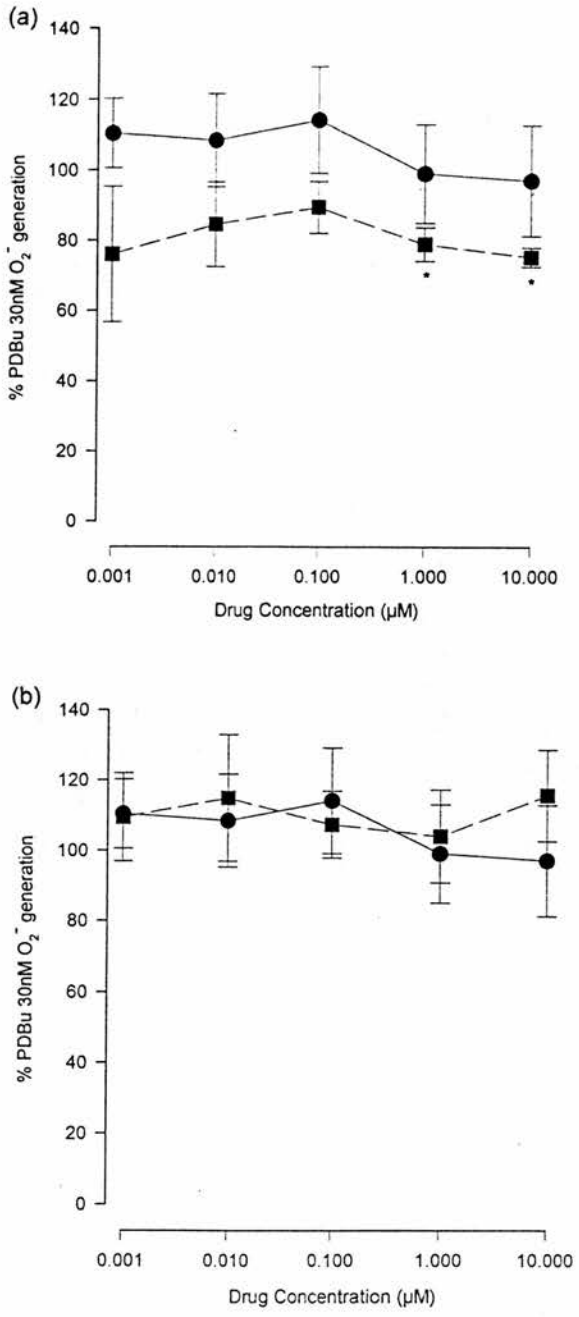

Figure 3.4.2.- (a) Effect of PGE_2 (●), n=7, and nocloprost (■), n=6, and (b) PGE_2 (●), n=7 and butaprost (■), n=6, on PDBu 30 nM induced superoxide anion generation in human monocytes. Nocloprost 10 μM induced a significant inhibition of 23 ± 7%.

*- $p<0.05$ compared to basal values, paired two-tailed Student *t*-test.

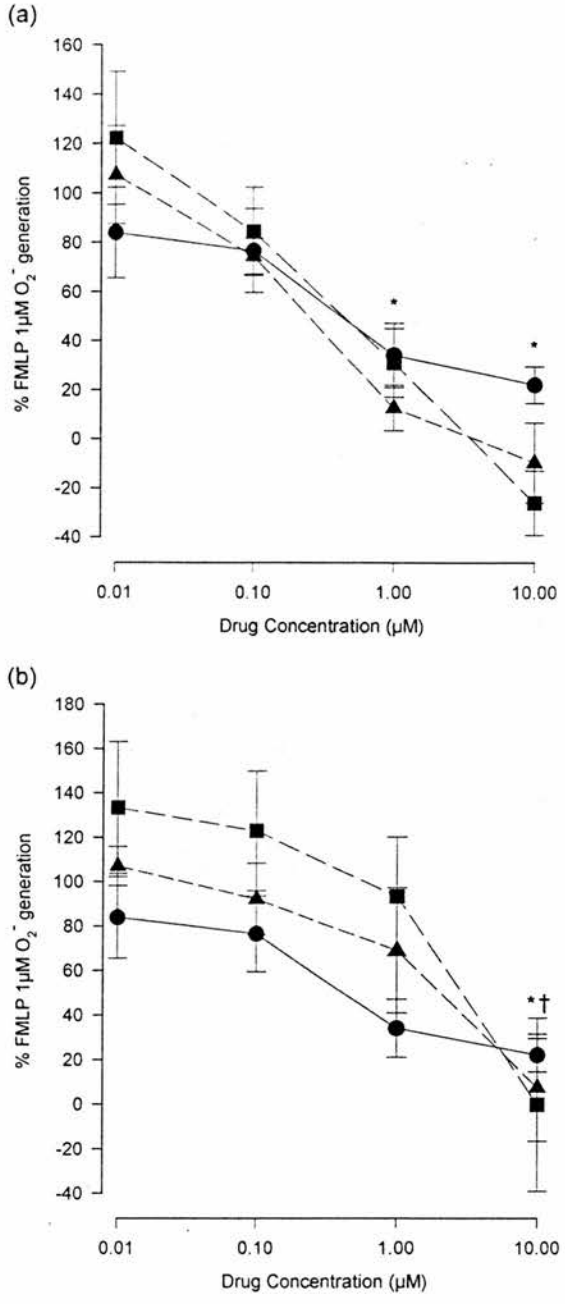

Figure 3.4.3.- (a) Effect of PGE_2 (●), n=5, 11-deoxy PGE_1 (■), n=4, and 16,16-dimethyl PGE_2 (▲), n=4, and (b) PGE_2 (●), n=5, butaprost (■), n=3, and AH13205 (▲), n=5, on FMLP 1 μM induced superoxide anion generation in human monocytes.

*- $p<0.05$ compared to basal values for PGE_2, 11-deoxy PGE_1, 16,16 dimethyl PGE_2 and AH13205, paired two-tailed Student *t*-test.

†- $p<0.05$ compared to basal values for butaprost, paired one-tailed Student *t*-test.

PGE_2, the standard agonist, concentration-dependently inhibited superoxide anion generation induced by FMLP, Figure 3.4.3a, Table 3.4.1. 16,16 dimethyl PGE_2 and 11-deoxy PGE_1 also concentration-dependently inhibited superoxide anion release, Figure 3.4.3a, and all agonists inhibited superoxide anion generation by greater than 75%.

Butaprost and AH13205 concentration-dependently inhibited FMLP-induced superoxide anion generation, but were not as potent as PGE_2, Figure 3.4.3b, Table 3.4.1. Interestingly, these compounds produced a greater percentage inhibition of superoxide anion generation than PGE_2.

Nocloprost was examined in this preparation and generated an unusual, but reproducible, inhibitory profile with a maximum inhibition of only 50%, Figure 3.4.4a, Table 3.4.1. Misoprostol, interestingly, inhibited superoxide anion generation with a lower EEC than PGE_2 of 0.3, Figure 3.4.4b, Table 3.4.1. and M&B 28767 induced a concentration-dependent inhibition of superoxide anion generation with a similar potency as PGE_2, EEC = 1.5, Figure 3.4.5a, Table 3.4.1.

GR63799X had no effect in this preparation whereas $PGF_{2\alpha}$ produced inhibition of superoxide anion generation , maximum inhibition of 53 ± 27%, but only at 10 μM, Figure 3.4.5b.

The EP_4-receptor antagonist AH23848B (10-100 μM) tended to potentiate the inhibition of superoxide anion generation by PGE_2, Figure 3.4.6a & b, however, these effects were non-significant. No effect was observed with AH23848B in the blank samples, or the FMLP induced superoxide anion generation, data not shown, which shows that AH23848B alone had no effect on the system. Interestingly, in these studies PGE_2 still generated a maximum inhibition of about 75% inhibition of FMLP induced superoxide anion, even when potentiated by AH23848B.

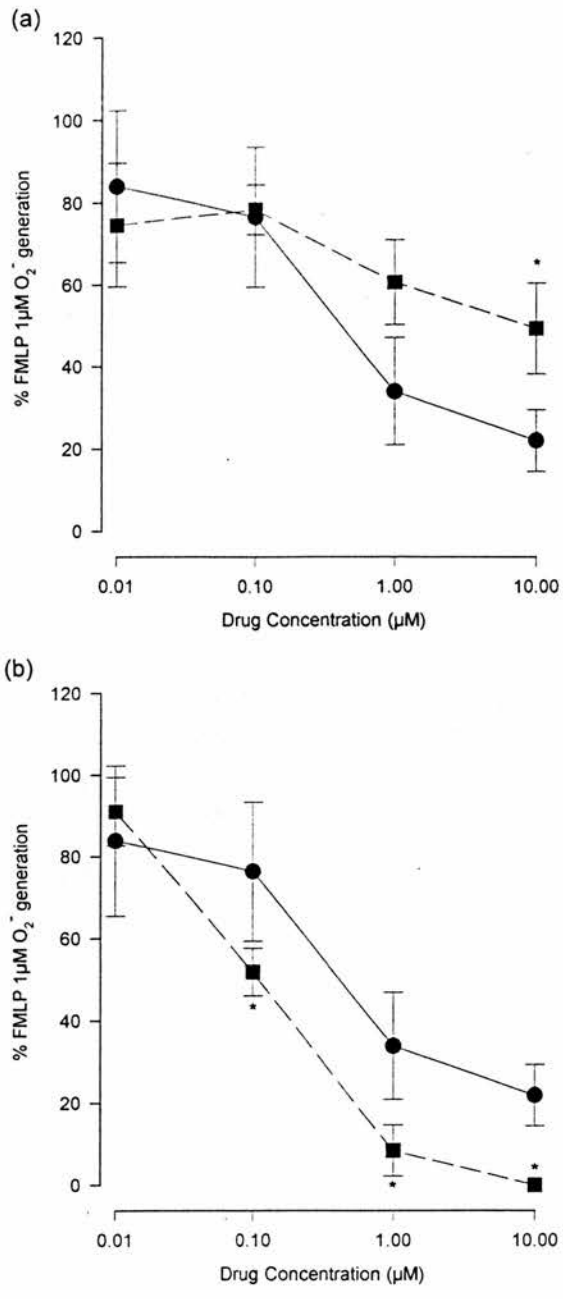

Figure 3.4.4.- (a) Effect of PGE_2 (●), n=5, and nocloprost (■), n=4, and (b) PGE_2 (●), n=5, and misoprostol (■), n=4, on FMLP 1 μM induced superoxide anion generation in human monocytes.

*- $p<0.05$ compared to basal values nocloprost and misoprostol, paired two-tailed Student *t*-test.

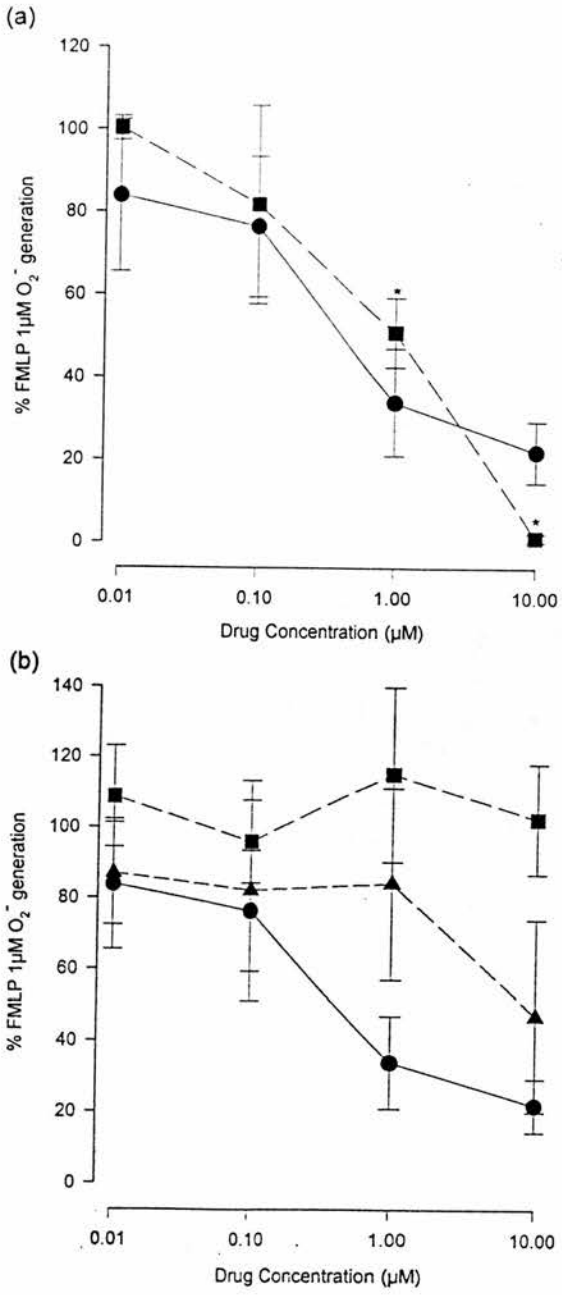

Figure 3.4.5.- (a) Effect of PGE_2 (●), n=5, and M&B 28767 (■), n=3, and (b) of PGE_2 (●), n=5, GR63799X (■), n=5, and $PGF_{2\alpha}$ (▲), n=4, on FMLP 1 μM induced superoxide anion generation in human monocytes.

*- $p<0.05$ compared to basal values for M&B 28767, paired two-tailed Student *t*-test.

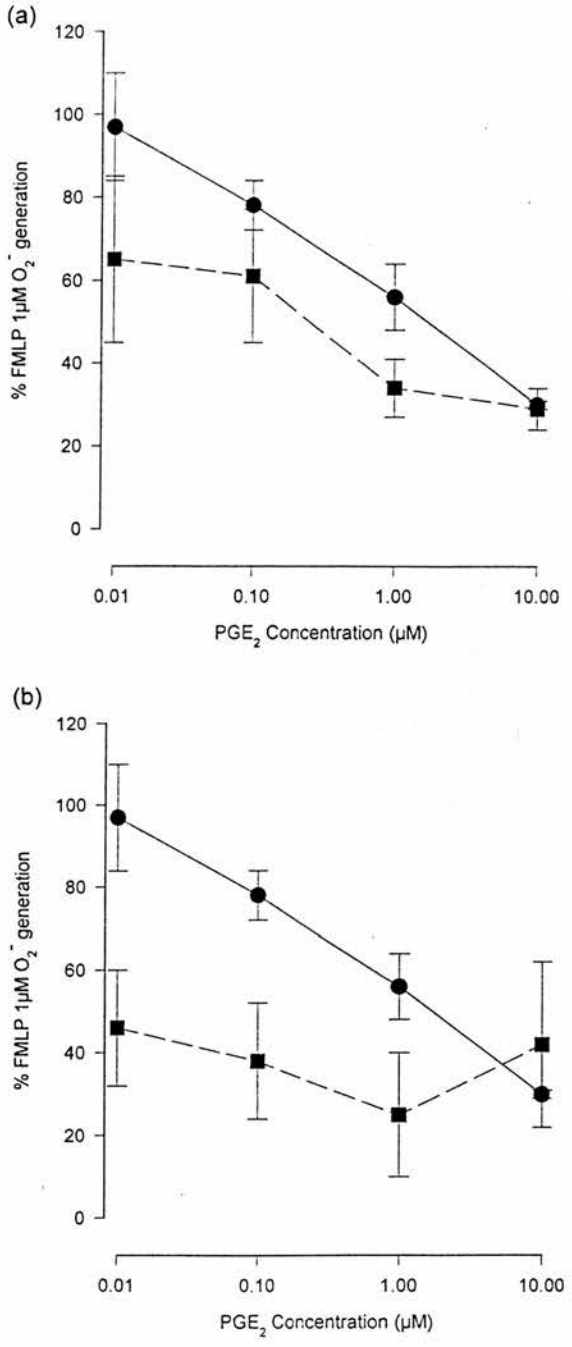

Figure 3.4.6.- Effect of the EP_4-receptor antagonist AH23848B 10 μM (■), n=4, and (b) AH23848B 100 μM (■), n=4, on the PGE_2 (●) concentration- response curves in human monocytes. AH23848B, alone, had no effect on the FMLP-induced O_2^- generation, data not shown.

$p>0.05$ with ANOVA for both graphs.

Analogue	**EC_{50} (μM)**	**maximum inhibition (%)**	**EEC PGE_2 = 1**
PGE_2	0.56 ± 0.20	78.1 ± 7.4	1.0
16,16 dime PGE_2	0.25 ± 0.19	97.1 ± 2.2	0.5
11-deoxy PGE_1	0.56 ± 0.35	99.5 ± 0.4	1.1
nocloprost	1.7 ± 1.1	50.8 ± 11.0	2.9
butaprost	3.6 ± 1.9	98.8 ± 0.6	6.5
AH13205	2.8 ± 1.7	92.6 ± 24.3	5.0
misoprostol	0.14 ± 0.06	98.3 ± 1.1	0.3
M&B 28767	0.84 ± 0.49	98.6 ± 1.1	1.5

Table 3.4.1. - The ability of EP agonists (0.01-10 μM) to inhibit O_2^- generation induced by FMLP (1 μM) (n≥4).

Second messenger studies

8-bromo cAMP (1 μM - 100 μM) produced no significant reduction in superoxide anion generation after stimulation with FMLP, however, in one experiment, Figure 3.4.7a, 100 μM caused a 44% reduction, Figure 3.4.7a-d.

Monocytes were preincubated for 5 min with IBMX before the addition of FMLP, and IBMX produced very large decreases in the basal absorbance as shown in Figure 3.4.8a-c, 2 out of 3 blank values are decreased in the presence of IBMX (0.25 mM). Analysed separately with the percentage inhibitions being relative to the individual curves, i.e. results without IBMX taken relative to the FMLP control without IBMX and results with IBMX expressed relative to the FMLP peak in the presence of IBMX, a non-significant potentiation of the PGE_2 inhibition can be seen. PGE_2 (10 nM) inhibited the FMLP peak by 15 ± 24% which was increased to a 33 ± 8% reduction in the presence of IBMX, Figure 3.4.9.

Rolipram (1 nM), a selective PDE IV isozyme inhibitor induced a decrease in the basal absorbance values like IBMX, as illustrated in the separate absorbance curves, Figure 3.4.10a-d. When each absorbance curve is analysed separately, as for IBMX, rolipram tended to potentiate the effects of PGE_2, Figure 3.4.11. Analysis with ANOVA, however, shows these effects to be non-significant, $p>0.05$.

SQ22536, an AC inhibitor, was studied in this preparation and SQ22536 (100 μM) caused a marked drop in the absorbance levels as seen for both IBMX and rolipram, Figure 3.4.12a-d. Analysed separately these data shows SQ22536 to significantly potentiate the effects of PGE_2, $p<0.05$ with ANOVA, Figure 3.4.13.

A PKA inhibitor H-89 was also investigated and H-89 (10 μM) and added 5 min before PGE_2 produced a significant potentiation of the response to PGE_2 10 nM, $p<0.05$, and a significant inhibition of PGE_2 1 μM, $p=0.05$, Figure 3.4.14.

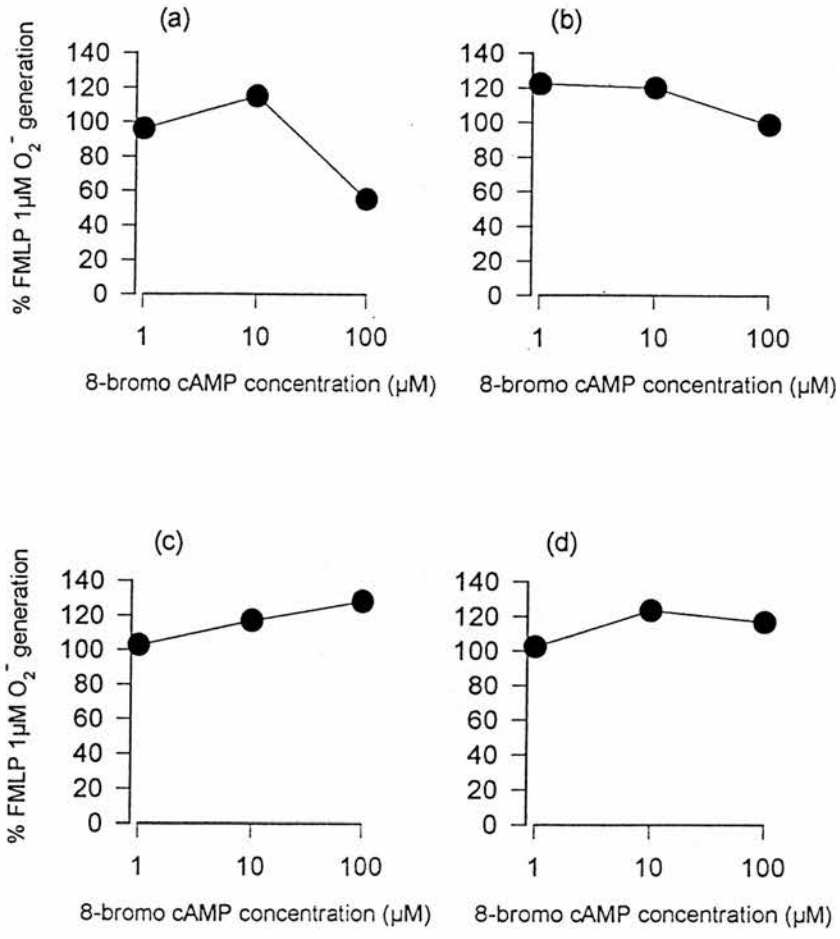

Figure 3.4.7a-d- Effect of 8-bromo cAMP (●) on FMLP 1 μM induced superoxide anion generation in human monocytes. Each figure represents an individual experiment.

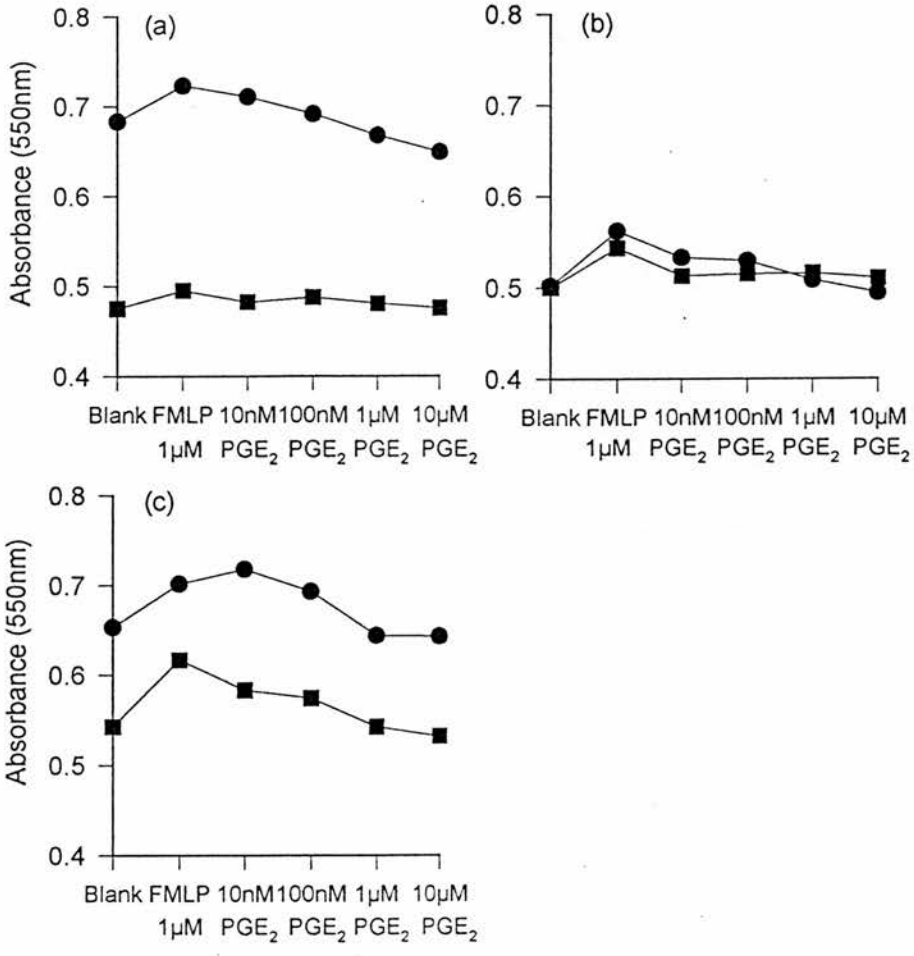

Figure 3.4.8a-c- Effect of PGE_2 on FMLP-induced superoxide anion generation in human monocytes in the absence (●) and presence (■) of IBMX (0.25 mM) expressed as raw absorbance (A_{550}) data. Each figure represents one experiment.

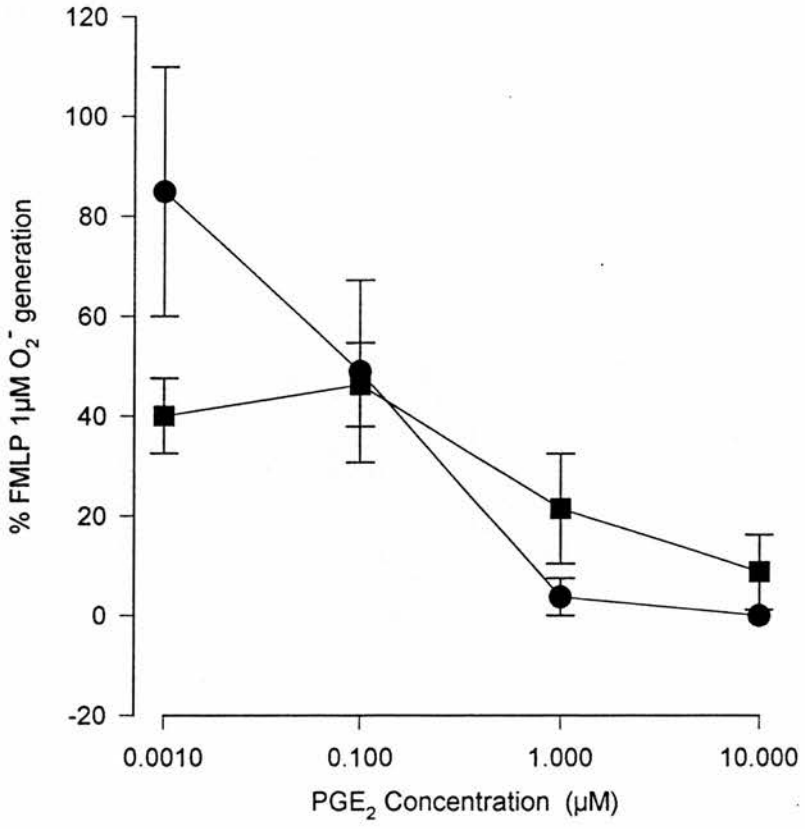

Figure 3.4.9.- Effect of PGE_2 on FMLP-induced superoxide anion generation in human monocytes in the absence (●) and presence (■) of IBMX (0.25 mM), n=3 ($p>0.05$ with ANOVA).

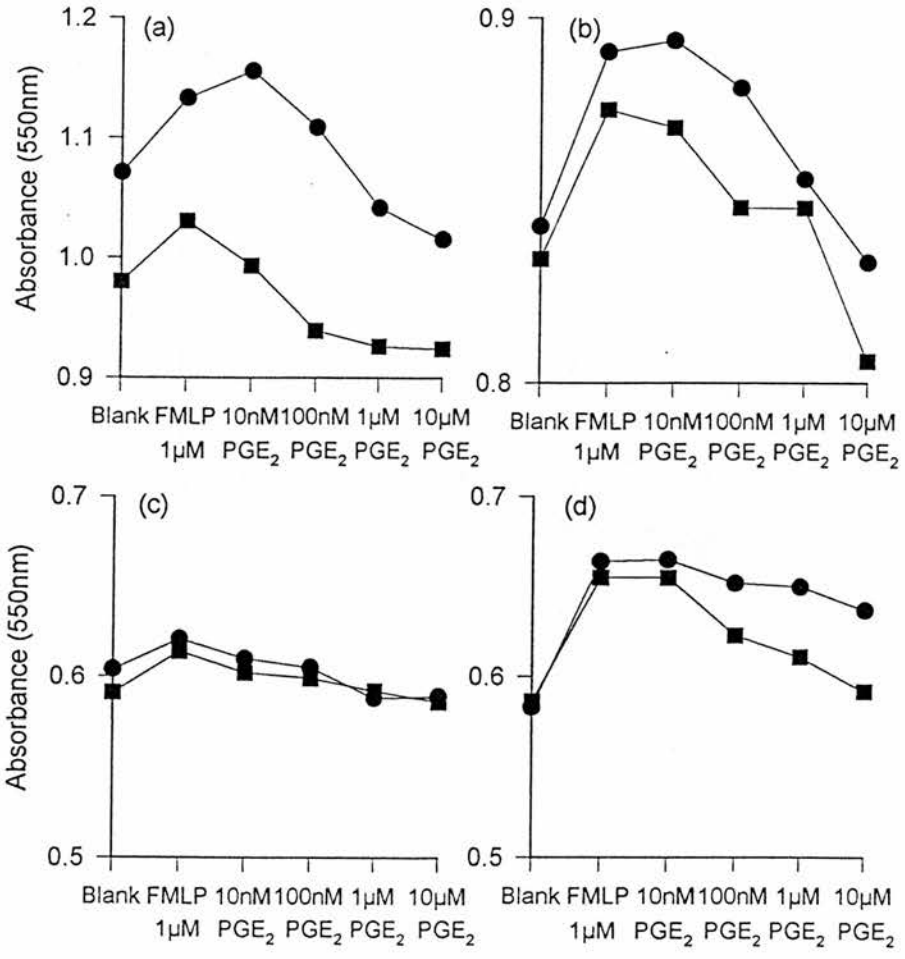

Figure 3.4.10a-d- Effect of PGE_2 on FMLP-induced superoxide anion generation in human monocytes in the absence (●) and presence (■) of rolipram (1 nM) expressed as raw absorbance (A_{550}) data. Each figure represents one experiment.

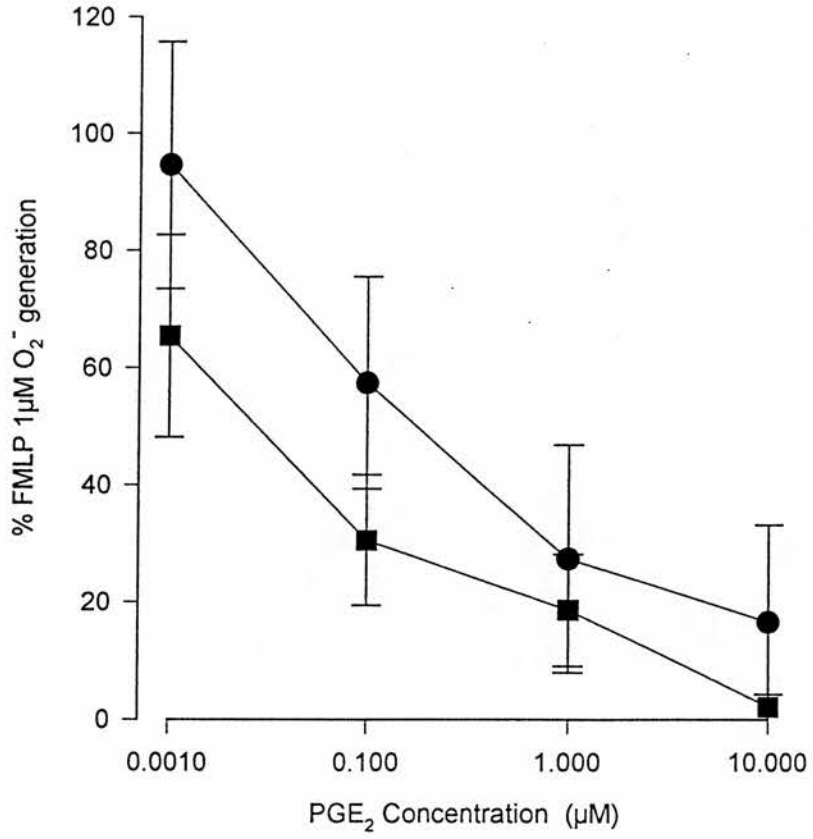

Figure 3.4.11.- Effect of PGE_2 on FMLP-induced superoxide anion generation in human monocytes in the absence (●) and presence (■) of rolipram (1 nM), n=4 ($p>0.05$ with ANOVA).

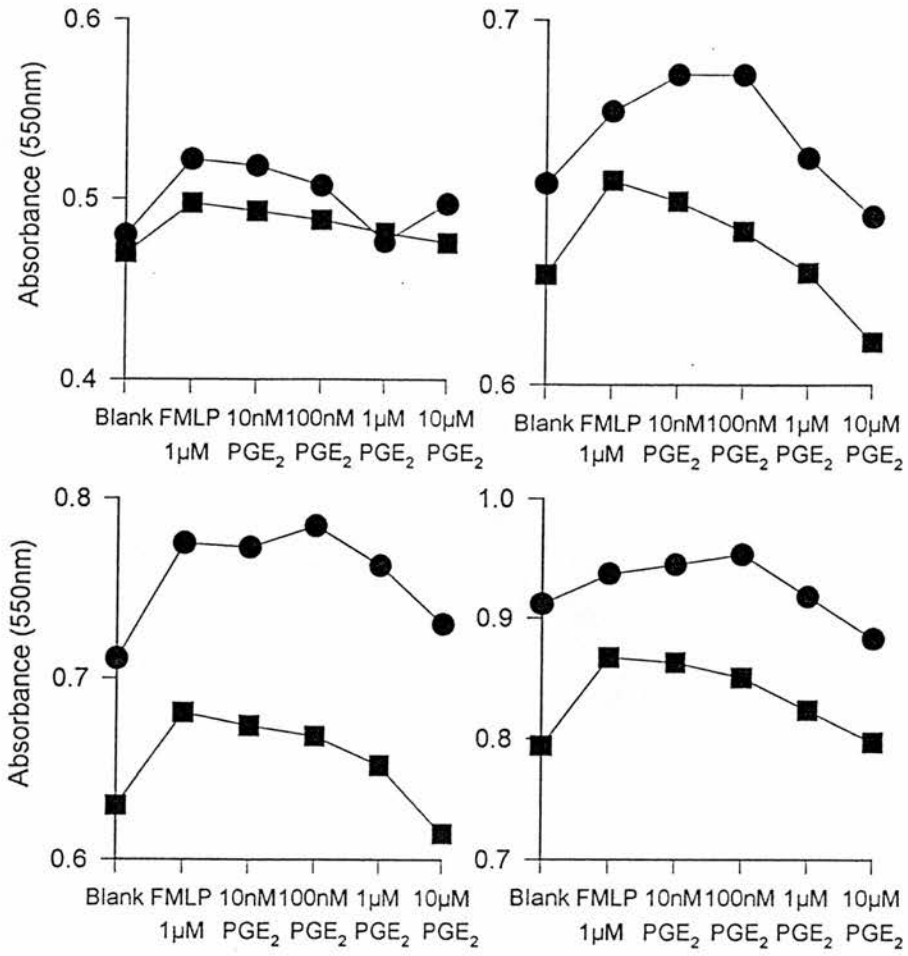

Figure 3.4.12a-d- Effect of PGE_2 on FMLP-induced superoxide anion generation in human monocytes in the absence (●) and presence (■) of SQ22536 (100 μM) expressed as raw absorbance (A_{550}) data. Each figure represents one experiment.

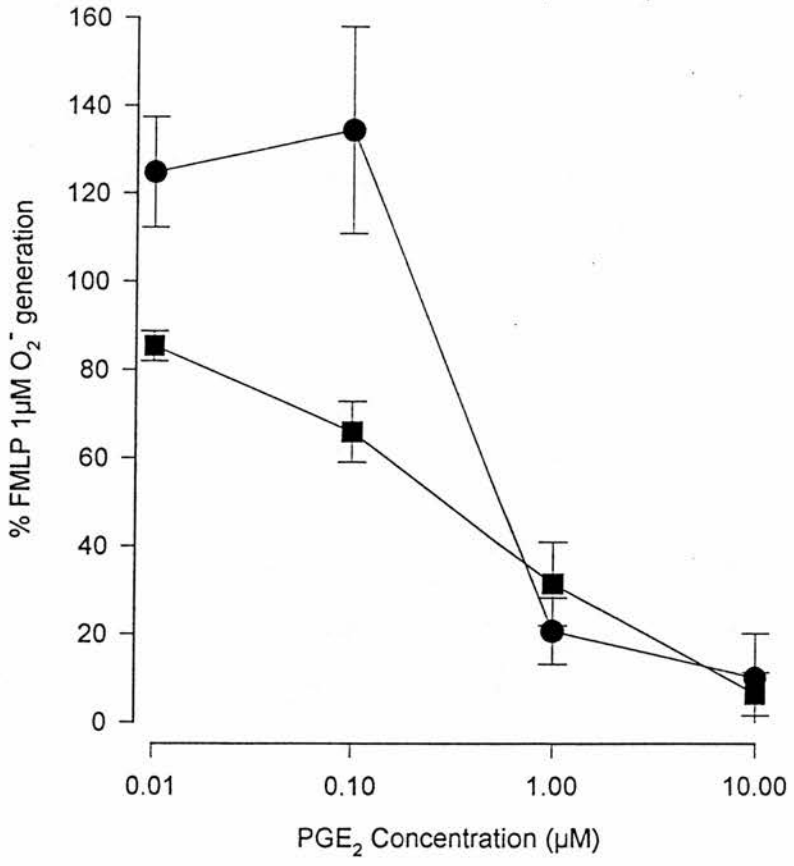

Figure 3.4.13.- Effect of PGE_2 on FMLP-induced superoxide anion generation in human monocytes in the absence (●) and presence (■) of SQ22536 (100 μM), n=4. SQ22536 significantly potentiated the effects of PGE_2, $p<0.05$ with ANOVA.

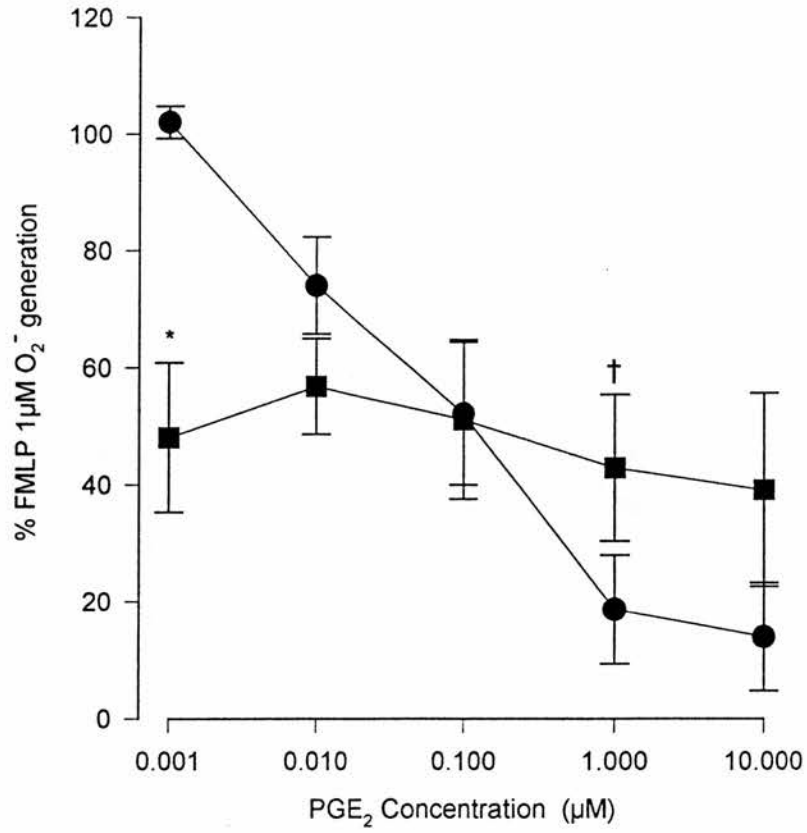

Figure 3.4.14- Effect of PGE_2 on FMLP-induced superoxide anion generation in human monocytes in the absence (●) and presence (■) of H-89 (10 μM), n=6.

*- $p<0.05$ compared to PGE_2, paired Student two-tailed *t*-test.

†- $p=0.05$ compared to PGE_2, paired Student one-tailed *t*-test.

Comparison of SOD and Blank wells

The optical density readings were compared for the SOD 90 U/ml cells, mean OD = 0.573 ± 0.022, against blank cells, mean OD = 0.584 ± 0.021, where equal volumes of HBSS were added instead of drugs. A two-tailed paired Student *t*-test of the two samples produced a $p<0.05$ ($n=28$) against the Null hypothesis that the cells were different and from these data it was decided to stop using SOD 90 U/ml as the total blank and used an HBSS blank instead.

3.4.4.- Discussion

The effects of PKC activators previously described as stimulants of superoxide anion generation were studied (Szelfer *et al.*, 1989; Yuo *et al.*, 1992; Wolfson *et al.*, 1993). No EP-receptor agonists caused any inhibition of PDBu stimulated superoxide anion generation, and only nocloprost produced a reduction of 21%. Thus, activation of the AC coupled EP-receptor present on human monocytes is insufficient a stimulus to inhibit direct PKC activated superoxide anion generation.

FMLP, whereas, produced concentration-dependent increases in superoxide anion generation which could be inhibited with a range of EP-receptor agonists. The finding that EP_2 and EP_4 receptor agonists such as 11-deoxy PGE_1 and 16,16-dimethyl PGE_2 (Milne *et al.*, 1995) and EP_2 selective agonists, butaprost and AH13205 were equipotent in this preparation suggests the involvement of EP_2 rather than EP_4-receptors. EEC values for these compounds at EP_2 and EP_4 containing preparations are shown in Table 3.4.2.

Misoprostol, an EP_2- and EP_3-receptor agonist also potently inhibited superoxide anion generation, being more potent than PGE_2 and M&B 28767, an EP_3-selective agonist, induced complete inhibition. These effects seen with M&B 28767 could be mediated via activation of EP_3 receptor splice variants but are more likely to be cross-reactivity with the AC coupled EP receptors expressed in the human monocyte as GR63799X, another selective EP_3-receptor agonist, produced no inhibition of superoxide anion generation. $PGF_{2\alpha}$ produced inhibition at its top concentration, but rather than acting via inhibitory FP receptors this effect is again probably mediated via cross-reactivity with the AC coupled EP-receptor in the human monocyte.

Analogue	EP_2[1]	EP_4[2]	human monocyte
11-deoxy PGE_1	13	2.0-7.3	1.1
16,16 dime PGE_2	9.4-20	2.8-7.3	0.5
butaprost	17	42-1200	6.5
AH13205	29	1120-11,000	5.0

Table 3.4.2. - Comparison of EEC values for a range of EP-receptor agonists at the EP_2 and EP_4 receptor, and in the human monocyte at inhibiting superoxide anion generation, PGE_2 = 1.0.

[1] - cat trachea (Dong *et al.*, 1986; Gardiner, 1986; Coleman *et al.*, 1988; Nials *et al.*, 1993)

[2] - PSV, RIT and cloned EP_4 receptor (Chapter 2.2; Louttit *et al.*, 1992b; Coleman *et al.*, 1994; Lydford & McKechnie, 1994; Nishigaki *et al.*, 1995)

The EP_4 receptor antagonist AH23848B was found to potentiate rather than block, inhibition of superoxide observed with PGE_2. This was an unusual observation and suggests that AH23848B has other activities along with its known TP and EP_4 receptor antagonism. Interestingly, AH23848B also potentiates inhibition of superoxide anion release with both PGE_2 and the non-prostanoid, 5'-N-ethylcarboxamido-adenosine in human neutrophils (Talpain *et al.*, 1995) as well as ECP release in a mixed polymorphonuclear cell population (E. M. Milne, personal communication). AH23848B has also been found to potentiate prostanoid-mediated responses in T-lymphocytes (D. F. Woodward, personal communication). AH23848B has also been demonstrated to be a partial agonist at the TP receptor (Brittain *et al.*, 1985; Lumley *et al.*, 1986) and may be, somehow, potentiating the effect of PGE_2. To ascertain if this effect is mediated by TP receptor activation, agonists, such as U-46619, need to be examined in this preparation.

As mentioned, the EP agonist data obtained in the cAMP studies on human monocytes do not correlate with the physiological effects observed *in vitro*. cAMP measurements suggested the involvement of the EP_4 receptor, whereas physiological inhibition of FMLP stimulated superoxide anion generation appears to be mediated via the EP_2 receptor. Misoprostol, for example, was shown to generate no cAMP in the monocyte, Figure 3.2.5a, and yet produced greater physiological inhibition of FMLP-mediated superoxide anion generation than PGE_2, Figure 3.4.4b, Table 3.4.1.

It is likely that the human monocytes contain a population of EP_2 receptors which mediate inhibition of FMLP-induced superoxide anion generation, and a population of EP_4 receptors positively coupled to AC which may or may not inhibit superoxide anion generation. Experiments using compounds which directly affect second messenger systems were carried out to try and clarify these conflicting data.

8-bromo cAMP is a cAMP mimetic, and was used to see if it could mimic the inhibition of superoxide anion generation observed with AC

activating agonists. No inhibition was observed on average but, when the experiments were examined separately, Figure 3.4.7a-d, it can be seen that 8-bromo cAMP 100 μM partially inhibited the FMLP-induced superoxide anion generation in one experiment only. Other investigators have looked at 8-bromo cAMP in the human monocyte and found it mimics the effect of other cAMP elevating agents (Sung *et al.*, 1991; Takii *et al.*, 1992) but does not induce complete inhibition. Interestingly, 8-bromo cAMP was inactive in the human neutrophils, whereas another cAMP mimetic, dibutyryl cAMP, was active and mimicked the effects of cAMP elevation (Dr. R. A. Armstrong, personal communication).

IBMX, a non-selective PDE isozyme inhibitor, was examined to look at the effect of PDE inhibition in the human monocyte. IBMX considerably reduced the absorbance in each experiment but did not affect the FMLP-induced superoxide anion generation, Figure 3.4.8a-c. When the absorbance data are analysed separately and pooled, Figure 4.3.9, IBMX potentiated the PGE_2 10 nM induced inhibition of superoxide anion generation suggesting some role for cAMP.

Rolipram, a selective PDE IV isozyme inhibitor, was investigated in this preparation as monocytes have been shown to express the PDE IV isozyme (Thompson *et al.*, 1976; Elliott *et al.*, 1989; Verghese *et al.*, 1995). Similarly to IBMX, rolipram caused a marked reduction in the baseline absorbance without affecting the increase in absorbance induced by FMLP, Figure 3.4.10a-d & 11. This was not due to any affect on the viability of the cells. Molnar-Kimber and co-workers (1993) measured the effects of selective PDE isozyme inhibitors on monocyte viability. They used vinpocetine (PDE-I), CI-930 and milrinone (PDE-III), rolipram and nitraquazone (PDE-IV) and zaprinast (PDE-V) and found that none of the inhibitors affected monocyte viability at 10 μM or lower concentrations. Taken together, however, rolipram did not significantly affect the PGE_2 inhibition of FMLP-induced superoxide anion generation, Figure 3.4.11. Overall, the results with the PDE inhibitors suggest only a minimal role for cAMP in the inhibition of superoxide anion generation by PGE_2.

The large decreases in baseline absorbance measured with IBMX and rolipram are unusual and it could be that the PDE inhibitors generate a physiological antagonism against an unknown endogenous stimulus in this system. However, SOD, which removes all endogenous superoxide anion, produced a similar absorbance to the blank wells and suggests that the monocytes are not already generating superoxide anion. Another possibility is that these compounds are affecting the redox potential of the cytochrome c since the decrease is observed in all samples. If these compounds are affecting the redox status of cytochrome c then the results are only valid as long as FMLP generates the same increase in absorbance, which has been shown to be the case.

It has already been demonstrated that PGE_2 elevates the levels of cAMP in macrophages derived from granuloma and that SQ22536, an AC inhibitor, markedly diminishes this rise in cAMP (Bonta *et al.*, 1981). In this study SQ22536 100 μM caused a marked drop in the absorbance levels similar to IBMX and rolipram, Figure 3.4.25a-d, however, analysed separately these data show a significant potentiation of the PGE_2 concentration-effect curve, Figure 3.4.13. If PGE_2 was causing inhibition of FMLP-induced superoxide anion generation via activation of AC then SQ22536 should have inhibited, rather than potentiated, the effects of PGE_2. PGE_2 may instead be inducing activation of a negative pathway of inhibition via an alternative route involving PKA activation at the same time as stimulating AC activity. This PKA linked pathway may then be potentiated in some way by SQ22536 leading to a physiological potentiation of PGE_2 mediated inhibition.

The PKA inhibitor H-89 was examined in this preparation to try and address the unusual result observed with SQ22536. H-89 10 μM significantly potentiated the inhibitory effect of PGE_2 10 nM. In contrast, H-89 significantly reduced the inhibition observed with PGE_2 1 μM. Therefore, PGE_2 may be mediating some of its inhibitory actions via PKA activation which could explain the lack of inhibition with SQ22536, but does not explain the potentiation seen with this

compound. However, these data are difficult to interpret and further studies are required with other PKA inhibitors.

In conclusion, it seems likely that both EP_2 and EP_4 receptors are present on the human peripheral blood monocyte and although the EP_4 receptors are positively coupled to AC, it is mainly EP_2 receptors which mediate inhibition of FMLP induced superoxide anion generation. The exact nature of the second messenger system which mediates PGE_2-induced inhibition of FMLP induced superoxide anion generation in the human monocyte needs further investigation to fully explain the results observed above.

3.5- Conclusion

The human monocyte has been shown to express the EP_4 receptor positively coupled to AC, whereas, PGE_2, and other EP-receptor agonists mediate inhibition of FMLP-induced superoxide anion generation via EP_2 receptors. No correlation was observed between cAMP generation and inhibition of physiological functions with EP_2 agonists such as butaprost, AH13205 and misoprostol producing no significant cAMP generation but inhibiting FMLP-induced superoxide anion release with a similar potency than PGE_2, Table 3.5.1.

Second messenger studies in the human monocyte indicated that PGE_2 was mediating the inhibition of superoxide anion generation by routes other than AC activation. Interestingly, it has been shown that FMLP activation of monocytes is accompanied by a rise in intracellular cAMP (Elliott *et al.*, 1986; Leonard *et al.*, 1987). If AC activation is involved in FMLP-induced superoxide anion generation, it could be that a component of PGE_2-induced inhibition might be via AC desensitisation.

The inhibitory effects of pulmonary surfactant have been demonstrated to be mediated both by activation of an inhibitory pathway involving cAMP and PKA, and inhibition of an activatory pathway involving PKC (Geertsma *et al.*, 1994). It could be that PGE_2 and other EP-agonists are acting in the same way which would help explain the lack of correlation with the cAMP data but not the results seen in the second messenger studies.

Another possible explanation for the results seen in the second messenger studies could depend on the activity of PGE_2, rather than the second messenger system. Only PGE_2 was used in the studies because it was the standard agonist but it may have been acting via other EP-receptors present in the human monocyte such as the EP_3 receptor. It has been observed in these studies that PGE_2, and nocloprost, induced inhibition of FMLP-induced superoxide anion generation but did not reach 100%. It could be that there is a component of EP_3 receptors present on the human monocyte that

Analogue	cAMP generation	inhibition of superoxide anion
11-deoxy PGE_1	3.1	1.1
16,16 dimethyl PGE_2	3.4	0.5
nocloprost	0.1	2.9
butaprost	>25	6.5
AH13205	>25	5.0
misoprostol	>25	0.3

Table 3.5.1. - Comparison of EEC values for a range of EP-receptor agonists at increasing intracellular cAMP levels and at inhibiting FMLP-induced superoxide anion generation in the human monocyte, PGE_2 = 1.0.

blocks physiologically the inhibition by PGE_2. However, evidence against this theory is that other broad spectrum agonists like 16,16 dimethyl PGE_2 inhibited completely FMLP activity. Further studies using AC activating agents to increase cAMP before the addition of EP-agonists are required to ascertain whether EP_3 receptors are also expressed in human monocytes.

In conclusion, the human monocyte contains a population of AC coupled EP receptors of the EP_4 receptor subtype and a population of EP_2 receptors mediating inhibition of FMLP-induced superoxide anion by a non-cAMP pathway. Further work with second messenger systems is required to fully elucidate the actions of PGE_2 on the human monocyte. Molecular biological techniques may also help elucidate the EP receptors endogenously expressed in the human monocyte. However, this suggests that there may be little scope to develop a monocyte-selective EP agonist since full inhibition requires EP_2 receptor selectivity similar to results observed with human neutrophils and eosinophils.

CHAPTER 4

General Discussion

The aims of this thesis were to investigate further the EP receptor subtype(s) present in the native CHO cell, RJV and PSV and, using the information obtained in these preparations, attempt to characterise which EP receptor subtype(s) were present in the human monocyte. The EP receptor subtype(s) present on the human monocyte were also studied to see if there was a functional inhibitory role which could be a target for drug development.

The results of these experiments indicate that the CHO and human monocyte both express EP_4 receptors positively coupled to AC. These are novel findings and indicate that the EP_4 receptor does couple to AC and increase intracellular cAMP.

The RJV was initially classified as an atypical EP_2 receptor containing preparation because of the inactivity of the selective EP_2 receptor agonist butaprost (Lawrence & Jones, 1992). Our studies confirm the inactivity of butaprost in this preparation as well as the inactivity of another EP_2 selective agonist AH13205. Unfortunately, however, no antagonism with AH23848B, an EP_4 receptor antagonist (Louttit *et al.*, 1992a), was observed. Comparison of the agonist potency profiles of the RJV with other EP_4 receptor containing preparations, Table 2.3.1. show similar profiles of $PGE_2 \geq$ 11-deoxy PGE_1 = 16,16 dimethyl PGE_2 > butaprost >> AH13205, and from this it could be suggested that the RJV is actually an atypical EP_4 preparation showing no antagonist activity with AH23848B. Further work with other published EP_4 receptor antagonists (Coleman *et al.*, 1994) may explain these results.

Interestingly, when the data obtained in the native CHO cell, RJV and PSV is compared with the EP_2 and EP_4 cloned receptors, and other EP_2 or EP_4 receptor containing preparations, is seems that endogenously the EP_2 and EP_4 receptors might be co-expressed. If this is correct then many published observations can be explained such as the potency of butaprost in the PSV but complete inability of butaprost to displace bound $[^3H]PGE_2$ from the cloned EP_4 receptor. As well as this, the observations that butaprost and AH13205 can act as antagonists in EP_4 receptor containing preparations may be explained by receptor co-expression. Our findings in the human monocyte suggest that both EP_2 and EP_4 receptors are expressed.

Interestingly, it seems that only EP_4 receptors are involved in cAMP generation by EP agonists whereas EP_2 receptors are mainly involved in mediating inhibition of FMLP-induced superoxide anion generation.

Studies with human monocytes, as described above, show these cells express the EP_4 receptor positively coupled to AC. Physiological investigations, however, indicate that the cells express the EP_2 receptor and the antagonist AH23848B potentiated the inhibitory effects of PGE_2, Chapter 3.4. Studies were carried out to investigate if cAMP was the second messenger mediating the inhibitory effects of PGE_2. The results were conflicting, and suggested that PGE_2 induced effects in the human monocyte other than EP receptor-mediated AC activation. It was observed that SQ22536, an AC inhibitor, potentiated the effects of PGE_2 and H-89, a PKA inhibitor mediated both stimulatory and inhibitory activity with PGE_2. From these results, and the observation that PGE_2 did not induce complete inhibition compared with other broad spectrum EP-agonists, it is likely that PGE_2 activates many second messenger systems in the human monocyte. Further work is required with other EP-agonists and modulators of second messenger pathways to elucidate the actions of PGE_2.

We have purified mRNA from human monocytes and sent the samples to Prof. John Regan in Arizona, USA, to investigate which EP-receptor subtypes were expressed in these cells using molecular biological techniques, see Appendix I. Unfortunately, Prof. John Regan and his team have been too busy to process our samples and we are currently attempting to set up these experiments in our own laboratories. It is hoped that these studies will confirm our findings and indicate which EP-receptor subtype(s) are being expressed by the human monocyte. Currently it is difficult to clearly indicate which systems are involved as most of the compounds used have activity at many EP-receptor subtypes and molecular biology may provide a valuable approach to support pharmacological experiments.

In conclusion, this work has demonstrated a role for PGE_2 and other EP-receptor agonists at inhibiting physiological functions of the human monocyte. The development of more selective agonists at prostanoid receptors may lead to the discovery of drugs which can aid in the treatment of inflammatory diseases.

This work highlights the limitations of the selective agonists at our disposal. Even with more selective agonists, previous data suggest that functional inhibition of the human monocyte is mediated by the same receptor subtype as for neutrophils and eosinophils. Unfortunately, these findings are not encouraging since the presence of the same functional receptors will limit the usefulness of such agents.

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APPENDIX I

mRNA EXTRACTION

DEPC-H_2O- diethyl pyrocarbonate (DEPC) 0.1% v/v is added to distilled water and left overnight at 4°C. The treated water was then autoclaved twice to break down any remaining DEPC.

0.3 M sodium acetate buffer pH 6.0- made up in DEPC-H_2O

3 M sodium acetate buffer pH 6.0- made up in DEPC-H_2O

GIT buffer- 94.53g guanidine isothiocyanate (GIT) (4M final), 1.67 ml 3M sodium acetate buffer pH 6.0, DEPC-H_2O to 200 ml and 1.67 ml 2-mercaptoethanol

CsCl buffer- 95.97g CsCl (5.7 M final), 0.83 ml 3 M sodium acetate pH 6.0 and DEPC-H_2O to 100 ml

Absolute ethanol

80% ethanol

mRNA extraction

1- 3 ml CsCl buffer was added to 6 tubes and ~7.5 ml of the GIT samples layered on top. The tubes were then centrifuged at 32,000 rpm for 18 hr at 20°C.

2- The supernatant was aspirated off to within 1 ml and the tube tipped horizontally to remove the remaining buffer. The pellet was then resuspended in 200 µl DEPC-H_2O by repeated pipetting before transferring to a 1.5 ml microfuge tube. 20 µl of 3M sodium acetate buffer was added and 440 µl 100% ethanol and the tubes left at -20°C for at least 30 min to precipitate the RNA.

3- Eppendorfs were centrifuged at 12,000 g for 10 min at 4°C. The supernatant discarded and 1 ml ethanol 80% added. The tubes were centrifuged again, as above, and the supernatant again discarded. The pellets were then dried at 37°C for 1-2 hr.

4- The pellets were resuspended in 100 µl DEPC-H_2O and 5 µl placed in 500 µl of H_2O for measurement. The samples can then be stored at -80°C under 3 volumes of absolute ethanol.

5- To measure the amount of RNA obtained the OD of the 500 µl sample was measured at 260 and 280 nm (pure RNA has a ratio of 2.0 260/280 nm and an OD_{260} of 1.0 equals an RNA concentration of 40 µg/ml).

Total RNA was extracted from two donors as described above. This was counted and a value of 0.023 was obtained for OD_{260} and 0.014 for OD_{280}.

This gives a ratio of 1.64 which implies some protein contamination of the RNA. This is likely caused at the initial stage when all the CsCl and GIT buffer must be adequately removed.

The OD_{260} value of 0.023 for the 5 µl sample diluted to 500 µl water means that there was 0.92 µg/ml, or 0.46 µg total in the 5 µl aliquot. So, in the stored sample of RNA, 495 µl, there was 45.54 µg RNA.

BUTTERWORTH HEINEMANN

Comparison of the EP Receptor Subtypes Mediating Relaxation of the Rabbit Jugular and Pig Saphenous Veins

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A fourth PGE receptor subtype, the EP_4 receptor, has recently been described in the pig saphenous vein (PSV). Similar to the EP_2 receptor, it mediates relaxation and is linked to stimulation of adenylate cyclase. The aim of this study was to determine whether or not the EP receptor present in the rabbit jugular vein (RJV), currently classified as an atypical EP_2 receptor, is of the EP_4 subtype. The relaxant activities of four EP_2 agonists, 11-deoxy PGE_1, 16,16-dimethyl PGE_2, butaprost, and AH 13205, on the RJV and PSV have been examined, and the effect of the EP_4 receptor antagonist AH 23,848B studied. The EP_2 agonists showed a similar order of potency on the two preparations. 11-Deoxy PGE_1 and 16,16-dimethyl PGE_2 were potent agonists on the EP_4 receptors of the PSV and on the RJV giving approximately equi-effective concentration ratios (EECs) of 2.0–6.6 and 2.8–9.9, respectively, compared to PGE_2 (EEC = 1), and so do not discriminate between EP_2 and EP_4 receptors. Butaprost was less active on these preparations (EEC 42–43) than on classical EP_2 receptors, and AH 13205 was much less active (EEC 3100–2780). While these results suggest that the EP receptors on the RJV are of the EP_4 subtype, this was not confirmed using the EP_4 receptors antagonist AH 23,848B.

Keywords: receptor; rabbit jugular vein; pig saphenous vein; relaxation

Introduction

It is proposed that the naturally occurring prostanoids PGD_2, PGE_2, $PGF_{2\alpha}$, PGI_2, and TXA_2 act on distinct receptor sites termed DP, EP, FP,

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IP, and TP receptors, to elicit a variety of biological responses. Such receptors were originally classified pharmacologically on smooth muscle preparations[1] using a wide range of selective agonists and, where possible, receptor antagonists. They have subsequently been cloned[2–5] and are rhodopsin-type receptors[6] with seven putative transmembrane domains, consistent with being G-protein–linked. In addition, the EP-receptor is further classified into four receptor subtypes, EP_1, EP_2, EP_3, and EP_4. Typically, EP_1 receptors mediate contraction (e.g., guinea-pig gastric fundus) and EP_2 receptors mediate relaxation of smooth muscle (e.g., rabbit ear artery, cat trachea), via activation of phospholipase C and adenylate cyclase, respectively. EP_3 receptor activation is more varied and includes inhibition of neuronally mediated contractions (e.g., guinea-pig vas deferens), potentiation of platelet aggregation, inhibition of gastric acid secretion, and contraction of some smooth muscle preparations (e.g., chick ileum), linked either to activation of phospholipase C or inhibition of adenylate cyclase. Identification of the most recently described subtype, the EP_4 receptor,[7] was based mainly on the use of the novel antagonist AH 23,848 (originally synthesised as a TP receptor antagonist), and analyzed using the pig saphenous vein (PSV) where PGE_2 was relaxant. Each of these receptor subtypes has now been cloned.[8–13]

The rabbit jugular vein (RJV) relaxes in response to PGE_2 and is currently classified as an atypical EP_2 preparation, based on the low activity of the EP_2 agonist butaprost.[14] The demonstration that another moderately potent and highly selective EP_2 agonist, AH 13205,[15] was only weakly active at EP_4 receptors of the PSV[7] led us to investigate further the nature of the EP receptor subtype(s) of the RJV.

The aim of this study was to compare relaxation of the isolated RJV and PSV by a range of EP agonists, 16,16-dimethyl PGE_2,[14] nocloprost,[16] 11-deoxy PGE_1,[17] butaprost,[18] and AH 13205.[15] Possible antagonism by AH 23,848, AH 6809,[19] and BWA 868C[20] has also been investigated.

Materials and Methods

Smooth Muscle Preparations

Rabbit Jugular Vein (RJV). Male New Zealand white rabbits (2–3 kg) were anesthetised with 30 mg/kg Sagatal (pentobarbitone) injected into a marginal ear vein. The external jugular veins were exposed, tied proximally to allow the vessels to fill before being tied distally, and the inflated sections (2–3 cm) removed, cleared of fat, and cut into rings 3–5 mm wide. Each ring was suspended in an overflow organ bath between two silver alloy hooks at 0.75–1.25 g tension and washed with Krebs' solution (composition, mM: NaCl 118, KCl 5.4, $CaCl_2$ 2.5, $MgSO_4$ 1.0, NaH_2PO_4

1.1, $NaHCO_3$ 25, glucose 10) containing 1 μM indomethacin, bubbled with O_2/CO_2 (95/5 v/v) and maintained at 37°C. Changes in tension were measured using Grass FT03 isometric transducers connected to a Maclab data acquisition system using Maclab v3.3 software.

After equilibration of the tissues for 1 h, the maximum contraction to 10 μM histamine was measured, and a concentration was selected, usually 2 μM (range 1–5 μM), that produced 60–80% of the maximum contraction. When a contraction had plateaued, cumulative doses of a PG analog were added to the organ bath, and each resulting relaxation was allowed to flatten before the next dose. All relaxant prostanoids were tested in the presence of GR 32191, 10 μM, to block any contractile action at TP receptors.[21]

Concentration-response curves for PGE_2 were carried out at the start and finish of each experiment. PGE_2 was used as the control in all experiments and complete relaxation of the precontracted tissues by PGE_2 was taken as the maximal relaxation (100%). All subsequent results were expressed as percentages of the PGE_2 maximum. Log concentration-response curves were plotted and the IC_{50} values determined using Kaleidagraph software. Each mean equi-effective concentration ratio (EEC) was calculated as the IC_{50} for the EP agonist/the IC_{50} for PGE_2.

Pig Saphenous Vein (PSV). External saphenous veins were removed from freshly culled Chinese Meishan pigs and set up as for the RJV, except that tissues were set at an initial tension of 2–3 g, phenylephrine (PE) was used as the contactile agent, and adenosine deaminase (1 U/mL) was added to limit fade. An initial concentration of 10 μM PE was used to determine the maximal contraction; subsequent concentrations which gave 60–80% of the maximum were 0.5–1 μM.

Statistical tests. Results are given as the mean ± SEM. Student's paired or unpaired 2-tailed *t*-tests were used for comparison of IC_{50} data.

Compounds. The following compounds were gifts which we gratefully acknowledge: nocloprost from Dr. E. Schillinger, Schering AG, Berlin; butaprost from Dr. P. Gardiner, Bayer, U.K.; AH 13205, AH 6809 and AH 23,848B from Dr. B. Bain, Glaxo, U.K.; BW A868C and L-NMMA from Dr. B. Whittle, Wellcome, U.K. PGE_2 and indomethacin were purchased from Sigma; 11-deoxy PGE_1 and 16,16-dimethyl PGE_2 were purchased from Cayman Chemicals.

Ethanolic stock solutions of the prostanoids (10–30 mM) were stored at −20°C and diluted with 154 mM NaCl for use. AH 23,848 B (1 mM) was made up daily by sonication in phosphate buffered saline (PBS, pH 7.15, Sigma).

Results

Rabbit Jugular Vein: Effect of Selective Antagonists

PGE_2 could relax the precontracted jugular vein completely (IC_{50} 8.73 ± 1.90 nM, Figure 1a). Preincubation with the EP_4 antagonist AH 23,848B (30 μM, 30 min) did not block the relaxant effect of PGE_2 (IC_{50} 9.09 ± 5.3 nM, n = 5, concentration-ratio (CR) 1.75 ± 0.67, P = 0.42, Figure 1a). Similarly, preincubation with the EP_1/DP antagonist AH 6809 (10 μM, 10 min) and the selective DP antagonist BW A868C (5.4 μM, 10 min) had no significant effect on the PGE_2 relaxation curve (IC_{50} = 5.5 ± 1.12 nM, n = 4, and 4.10 ± 2.31 nM, n = 3, CR 0.78 ± 0.33 and 1.75 ± 0.84, P = 0.34 and 0.77, respectively). CRs of 10,000 and 10 would have been predicted with BW A868C[20] and AH 6809,[19] respectively, on the basis of their reported pK_Bs of 9.3 and 6.0 for DP receptors, if relaxation induced by PGE_2 resulted from an interaction with DP receptors.

Effect of Selective Agonists

A comparison of the ability of various agonists with activity at EP_2 receptors to relax the RJV is shown in Figures 1b and 1c. The rank order of potency is $PGE_2 \geqslant$ nocloprost > 11-deoxy PGE_1 > 16,16-dimethyl PGE_2 > butaprost ⩾ AH 13205. Table 1 gives IC_{50} and EEC values of agonists. 11-Deoxy PGE_1 achieved a maximal relaxation of 73.1 ± 7.24%, whereas other EP_2 agonists of similar potency, 16,16-dimethyl PGE_2 and nocloprost, gave between 90% and 104% of the PGE_2 maximum.

Endothelium Dependency

N^G-monomethyl-L-arginine (L-NMMA), a stable NO synthase inhibitor,[22] was used in the RJV preparation to examine the dependency of an intact endothelial layer. At a concentration of 100 μM (15 min preincubation), L-NMMA had no significant effect (P = 0.33) on relaxation induced by PGE_2 in the RJV (PGE_2 IC_{50} 9.48 ± 4.07 nM; PGE_2 + L-NMMA IC_{50} 6.18 ± 2.11 nM, Figure 2).

Pig Saphenous Vein: Effect of Selective Antagonists

The PSV was slightly more sensitive than the RJV to relaxation induced by PGE_2 (IC_{50} 2.05 ± 0.93 nM, P = 0.05; Figure 3a). Preincubation with the EP_4 antagonist AH 23,848B (30 μM, 30 min) shifted the concentration-response curve for PGE_2 to the right, (IC_{50} 6.48 ± 2.91 nM compared to 2.67 ± 1.89 nM, n = 5, giving a CR of 5.94 ± 1.45, P = 0.048, and a pA_2 of 5.27). Preincubation with the EP_1/DP antagonist AH 6809 (10 μM, 10 min) did not antagonize PGE_2 (IC_{50} 3.28 ± 1.39 and 3.85 ± 3.09 nM) in the presence and absence of AH 6809, P = 0.87.

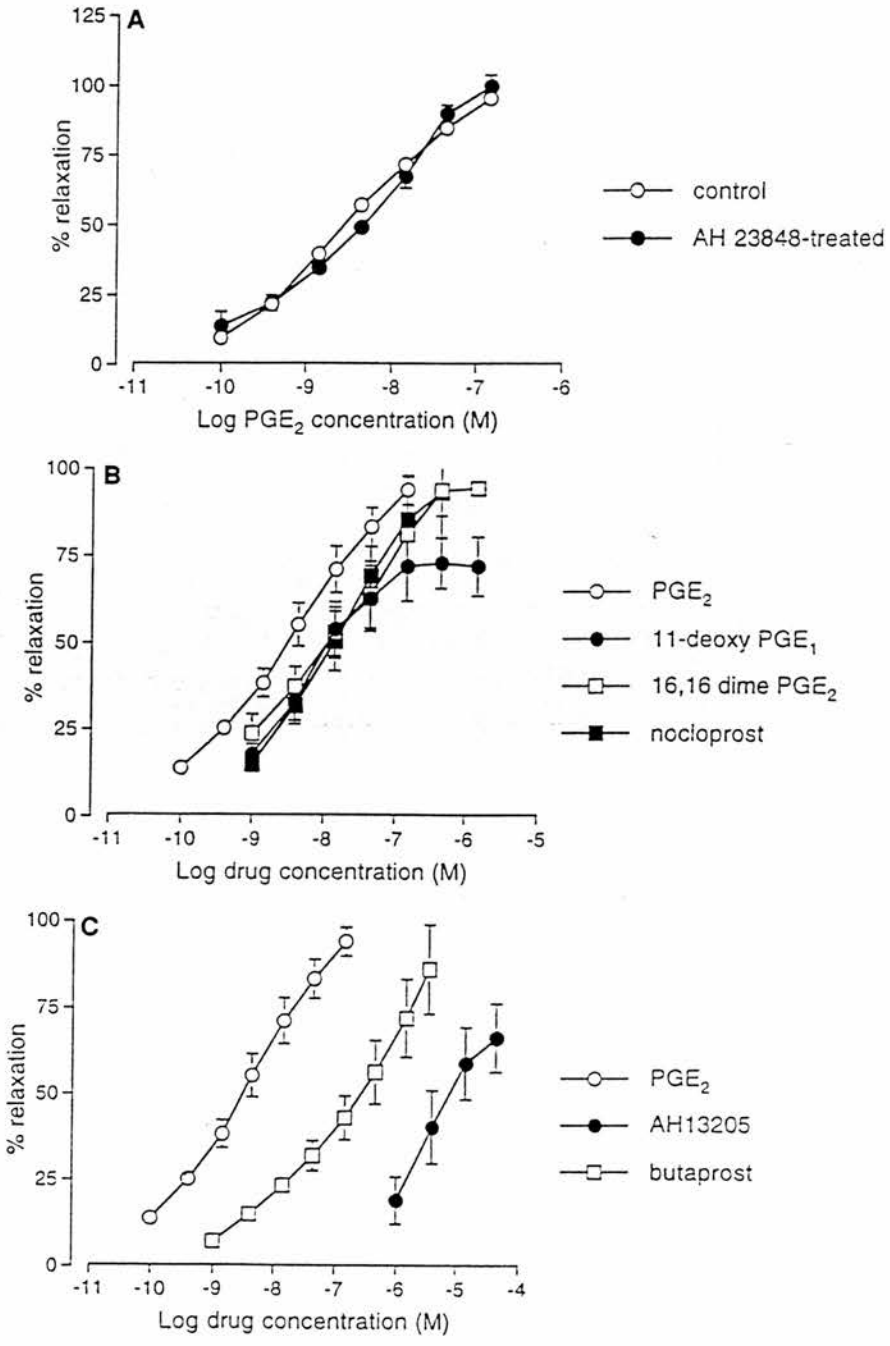

FIGURE 1. Log concentration-response curves for the effects of PGE_2 and PGE analogs on the RJV precontracted with histamine. Relaxation is expressed as a percent of the maximum relaxation induced by PGE_2 (100%). (a) PGE_2 in the presence and absence of the EP_4 receptor antagonist AH 23,848B (30 μM, 30 min, n = 4). (b) PGE_2, nocloprost, 11-deoxy PGE_1, and 16,16-dimethyl PGE_2. (c) PGE_2, butaprost, and AH 13205. Results are expressed as means ± SEM.

TABLE 1. Inhibitory concentrations (IC_{50}) and equi-effective concentrations (EEC, PGE_2 = 1) for the pig saphenous vein (PSV) and the rabbit jugular vein (RJV)

	IC_{50}		EEC	
	PSV	RJV	PSV	RJV
PGE_2	2.05 ± 0.93 nM	8.73 ± 1.90 nM	1.0	1.0
11-Deoxy PGE_1	3.17 ± 1.41 nM	61.32 ± 52.91 nM	2.0	6.6
16,16-Dime PGE_2	3.37 ± 1.23 nM	87.03 ± 34.18 nM	2.8	9.9
Butaprost	83.9 ± 21.8 nM	381.0 ± 240.5 nM	42	43
AH13205	6.36 ± 2.58 μM	20.26 ± 8.30 μM	3100	2780

IC_{50} = value ± SEM ($n \geq 4$).

Effect of Selective Agonists

A comparison of the ability of various agonists with activity at EP_2 receptors to relax the PSV is shown in Figures 3b and 3c. The rank order of potency is $PGE_2 \geq$ 11-deoxy $PGE_1 \geq$ 16,16-dimethyl $PGE_2 >$ butaprost $\gg$ AH 13205. Relaxation of at least 95% of the maximum induced by PGE_2 was achieved by all the EP agonists tested. IC_{50} values and EECs are given in Table 1.

Discussion

The purpose of these experiments was to further characterize the EP receptor subtype(s) present in the RJV. The PSV has been used as a known

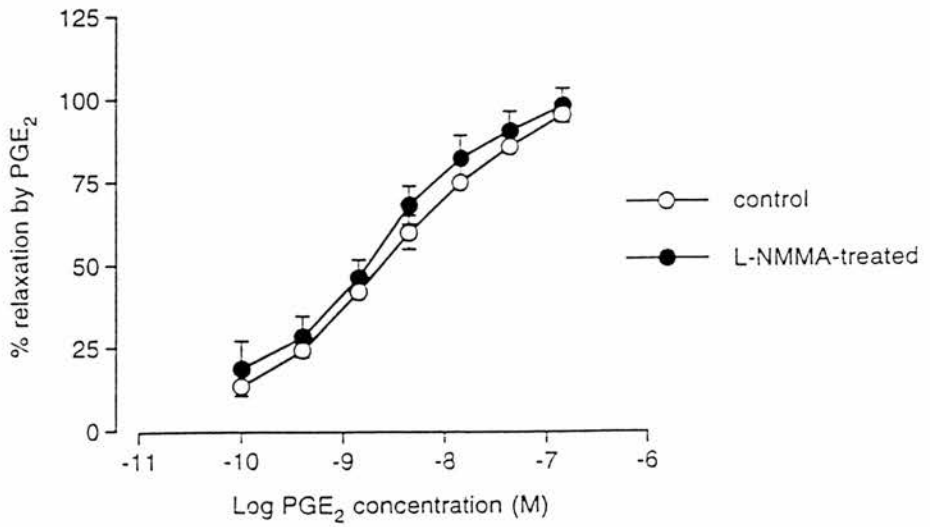

FIGURE 2. Relaxation of precontraction of the RJV by PGE_2 in the presence and absence of the inhibitor of nitric oxide synthesis L-NMMA (100 μM, 15 min, n = 8).

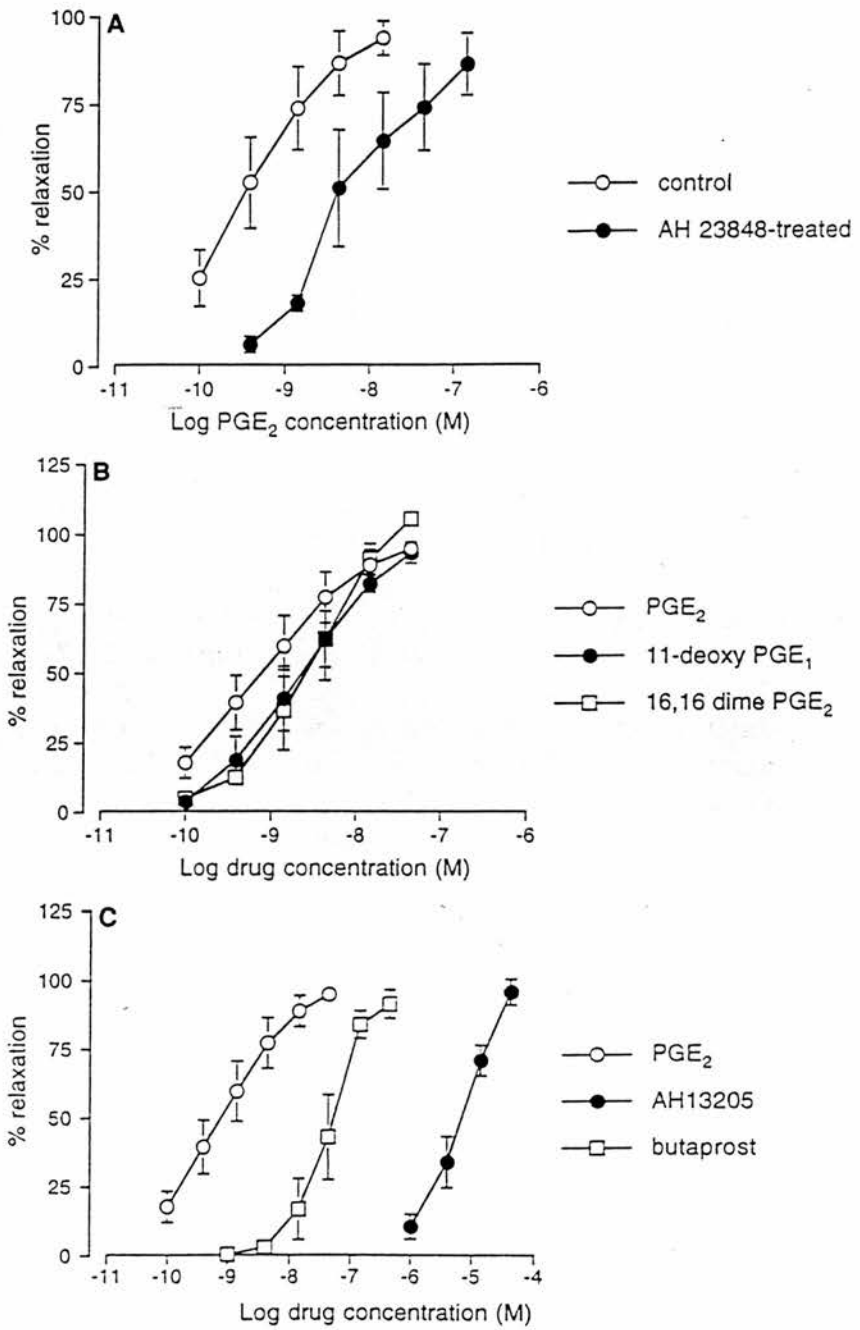

FIGURE 3. Log concentration-response curves for the effects of PGE_2 and PGE analogs on the PSV precontracted with phenylephrine. Relaxation is expressed as a percent of the maximum relaxation induced by PGE_2 (100%). (a) PGE_2 in the presence and absence of the EP_4 receptor antagonist AH 23,848B (30 μM, 30 min, n = 4), P = 0.048. (b) PGE_2, 11-deoxy PGE_1, and 16,16-dimethyl PGE_2. (c) PGE_2, butaprost, and AH 13205. Results are expressed as means ± SEM.

EP_4-receptor–containing preparation to confirm the activity of the antagonist AH 23,848 and to determine the activities of four commonly used EP agonists at EP_4 receptors. Quantification of agonist action ideally requires estimation of both affinity and efficacy, since these measurements explain in more detail the IC_{50} values. The most reliable method for determining these variables involves irreversible receptor blockade,[23] but unfortunately no irreversible antagonist at prostanoid receptors is available. Therefore in this study the comparison of the relative potencies of four EP agonists, and possible block by receptor antagonists where available, have been used in an attempt to characterize the receptor subtypes involved.

Rabbit Jugular Vein

The RJV is known to contain a heterogeneous population of prostanoid receptors,[24,25,14] namely thromboxane (TP) receptors mediating contraction (blocked here with the TP receptor antagonist GR 32191) and receptors for PGE_2, PGD_2, and PGI_2 mediating relaxation. Similar to these other authors, we found that the selective DP receptor antagonist BW A868C failed to antagonize the response to PGE_2 at a concentration 10,000 times greater than its pK_B at DP receptors. Similarly, AH 6809 which is less selective and has weaker DP receptor block, failed to antagonize the PGE_2 response. This suggests that PGE_2 does not mediate relaxation via an interaction at DP receptors. The exclusion of an interaction of PGE_2 at IP receptors has not been addressed here as there are no selective IP receptor antagonists available, but this mechanism seems unlikely as PGE_2 is more potent on the RJV than are PGI_2 or selective IP analogs.[24,14] Furthermore, as would be expected, this EP receptor is on the smooth muscle and so relaxation is not endothelium-dependent. Thus, the NO synthase inhibitor L-NMMA did not significantly affect relaxation induced by PGE_2 (Figure 2). This was also seen when the tissue was denuded of endothelium ($n = 2$, data not shown).

Agonist data in Table 1 confirm the relatively low potency of butaprost on the RJV.[14] However, although the IC_{50} values for butaprost are similar in the two studies, 381.0 ± 240.5 nM here compared to 199.5 nM, the EEC values of 43 and 685 are different and reflect the potency of PGE_2 in the two studies (IC_{50}s of 8.73 ± 1.9 nM and 0.46 nM, respectively).

The reasons for the difference in sensitivity of the RJV to PGE_2 in these two studies are still not entirely clear, but we have a few considerations which might help to explain this disparity:

1. The concentration of histamine used in our studies, while submaximally effective, was usually 2–5 μM, whereas Lawrence and Jones[14] typically used 1 μM. Relaxation by PGE_2 of the histamine-induced contraction is a form of functional antagonism, so perhaps a smaller contraction

is easier to block than a larger contraction.[26] This can be seen to a limited extent in Figure 4, where significantly more relaxation was observed with 0.14 μM and 0.45 μM PGE_2 using 2 μM compared with 5 μM histamine ($P = 0.04$).

2. Definition of the maximal relaxation induced by PGE_2 is different in the two studies. Lawrence and Jones[14] used relaxation by PGE_2 of the contractile response to histamine as 100% relaxation, whereas we have used the complete relaxation induced by PGE_2 as 100%. Since the tissues are already under tension before the addition of histamine, PGE_2 usually relaxes the tissue to below the basal tension prior to the addition of histamine (Figure 5). Calculating the IC_{50} value for PGE_2 by these two methods in the same preparations gives values of 7.9 ± 2.4 nM using the observed relaxation induced by PGE_2 as the maximum, and 3.8 ± 1.5 nM when relaxation of the precontraction induced by histamine is taken as the maximum ($P = 0.09$, n = 4). While these points may partly explain the lower sensitivity to PGE_2 in our experiments compared to those of Lawrence and Jones, this should not affect the EEC values calculated in both studies and so does not explain the greater sensitivity to butaprost seen here. The EECs of 6.6 and 9.9 for 11-deoxy PGE_1 and 16,16-dimethyl PGE_2 found in this study (Figures 1b and 1c) compare more favorably with those of 1.4 and 2.1 found by Lawrence and Jones. AH 13205 had not been tested previously on the RJV, and was only weakly active (IC_{50} = 20.26 ± 8.30 μM). Similarly, nocloprost had not been tested previously, and was a potent agonist (IC_{50} 4.9 ± 3.1 nM).

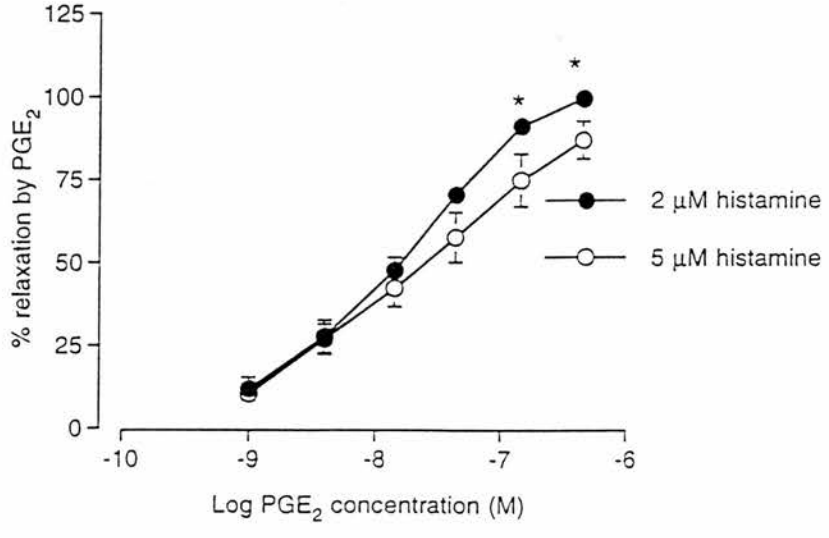

FIGURE 4. Log concentration-response curves for PGE_2 on the PSV, precontracted by 2 or 5 μM histamine, n = 4. PGE_2 (1 nM–10 μM) was a more effective relaxant agent with the lower concentration of histamine than at the higher concentration *$P < 0.05$.

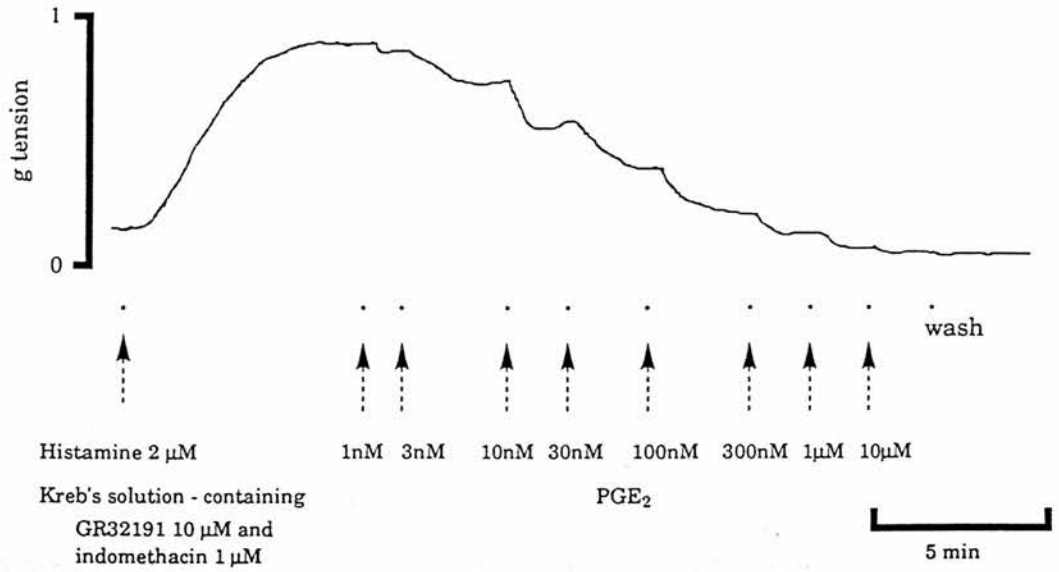

FIGURE 5. Example of a typical RJV trace. Contraction was induced by 2 μM histamine and the preparation relaxed by cumulative doses of PGE_2. The preparation relaxed to below the original set tension.

Pig Saphenous Vein

The PSV has been confirmed to contain EP_4 receptors, with the antagonist AH 23,848B (Figure 3a) giving a pA_2 of 5.27 which is similar to the 5.4 previously described.[7] A pA_2 value measures antagonist potency[27] but does not give any information about the nature of antagonism, as to whether or not it is competitive. For competitive antagonists, pA_2–pA_{10} should equal log 9, and then the pA_2 value can be taken to be equal to the pK_B. However, it is not possible with a low affinity antagonist such as AH 23848 to look for greater block, as this would require using concentrations of antagonist higher than 30 μM, and so the K_B value for AH 23,848 cannot be determined for EP_4 receptors. No block was observed with the DP/EP_1 receptor antagonist AH 6809, suggesting that PGE_2 does not mediate relaxation by an interaction with DP receptors in the PSV. The IC_{50} determined for PGE_2 on this preparation is 2.05 ± 0.93 nM, which agrees well with the value of 2.5 nM determined by Coleman,[28] but is 10-fold higher than he originally reported.[7] The PSV was used to test the EP_4 activity of four commonly used EP_2 receptor agonists (Figure 3b). Relative activities of these compounds on the PSV were $PGE_2 \geqslant$ 11-deoxy PGE_1 = 16,16-dimethyl PGE_2 > butaprost ⩾ AH 13205. The profile of the relative activities of the EP agonists on the RJV and PSV (Table 1) is very similar. Butaprost was less active in both preparations than would be predicted from its EP_2 receptor activity, but was more active than the EP_2 agonist AH 13205. 11-Deoxy PGE_1 and 16,16-dimethyl PGE_2 were potent ago-

nists on both EP_2 and EP_4 receptors and therefore are not useful agonists for discriminating between these receptor subtypes.

The agonist data would suggest that the RJV is a preparation containing EP_4 receptors, but in contrast no antagonism was observed with AH 23,848B on this tissue (Figure 1a). The reason for this lack of antagonism is unclear, although it has been documented elsewhere that TP antagonists have lower affinity for thromboxane receptors in the rabbit than for other TP receptor-containing preparations,[29] and this may be true here for EP receptors. Unfortunately it is difficult to test AH 23848B at concentrations above 30 μM in the organ bath. Experiments with the more recently described EP_4 receptor antagonist AH 22,921[28] may help to resolve the nature of the EP receptor subtype in the RJV.

In conclusion, the EP receptor agonists 11-deoxy PGE_1, 16,16-dimethyl PGE_2, butaprost, and AH 13205 show similar activities on the RJV and on the EP_4 receptors of the PSV. Butaprost was less active on these preparations (EEC 42–43) than on classical EP_2 receptors, but AH 13205 was dramatically less active (EEC 3100–2780). While the results with the agonists suggest that the EP receptors on the RJV are of the EP_4 subtype, this was not confirmed using the EP_4 receptor antagonist AH 23,848B.

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